A STUDY ON THE INTRIGUING PATHOGENIC RELATIONSHIP BETWEEN LIPOPROTEIN (a) AND TRANSFORMING GROWTH FACTOR-β IN ATHEROSCLEROTIC PERIPHERAL VASCULAR DISEASE

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BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **A STUDY ON THE INTRIGUING PATHOGENIC RFELATIONSHIP BETWEEN LIPOPROTEIN (a) AND TRANSFORMING GROWTH FACTOR-β IN ATHEROSCLEROTIC PERIPHERAL VASCULAR DISEASE** is the original bonafide work done by **Dr.VEENA JULIETTE.A**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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ABBREVIATION

PVD	-	Peripheral Vascular Disease
ABPI	-	Ankle Brachial Pressure Index
VLDL	–	Very Low Density Lipoprotein
LDL	–	Low Density Lipoprotein
HDL	–	High Density Lipoprotein
Lp(a)	-	Lipoprotein (a)
TGF-β	-	Transforming Growth Factor-β
TNF-α	–	Tumour Necrosis Factor-α
DM	–	Diabetes Mellitus
SMK	–	Smoking
ALC	–	Alcoholism
WT	–	Weight
HT	–	Height
BMI	–	Body Mass Index
CHOL	–	Cholesterol
TGL	–	Triglyceride
ER	–	Endoplasmic reticulum
IL	-	Interleukin
TLR	-	Toll Like Receptor
NF-κB	-	Nuclear Factor κB
IFN	-	Interferon

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INTRODUCTION

INTRODUCTION

Peripheral Vascular Disease (PVD), or Peripheral Arterial disease, is one of the major causes of morbidity and mortality in the Indian population, the prevalence increasing in elderly and diabetic people. Chronic lower extremity ischemia is the most common cause of loss of walking ability attended by a vascular surgeon and is associated with a constellation of disorders affecting the entire system[1]. Atherosclerotic PVD is a prototype of chronic systemic atherosclerosis and is characterized by arterial stenoses and occlusions in the peripheral arterial bed of lower limbs. The various risk factors include Age, DM, Smoking, hypertension, hyperlipidemia, hyperhomocysteinemia etc.

Atherosclerosis is a chronic inflammatory disease. Cardiovascular atherogenicity is the major cause of mortality around the world, though it can effect all the medium and large sized vessels in the body. Dyslipidemia is a major risk factor for the development and progression of atherosclerosis, along with the life style and co morbid conditions like Diabetes mellitus. The major lipids of human body are phospholipids, cholesterol, triglycerides and cholesteryl esters. These insoluble lipids are transported through the blood as lipoprotein complexes of lipids and one or more of specific proteins called apolipoproteins. By actively exchanging certain lipids and apolipoproteins with each other, the lipoproteins are synthesized and degraded at a constant rate. Among lipoproteins, low density lipoproteins (LDL) has 75% lipid and 25% proteins. Modification of LDL, mainly Oxidation, plays a key role in the evolution of atherosclerosis. High density lipoprotein (HDL) has a protective role in atherosclerosis, because it is involved in reverse cholesterol transport and helps to excrete cholesterol by

the liver. Another role of HDL in protection against atherosclerosis is by inhibiting the oxidative modification of LDL.

Lipoprotein(a) is a genetically determined, cholesterol rich plasma lipoprotein which is a risk factor for atherosclerosis. High Lp(a) concentration represents an indicator of risk for cardiovascular disease, especially when serum LDL-cholesterol or Apo B are elevated. Lp(a) levels are found to be resistant to standard lipid lowering therapy, with the exception of Niacin.

Lp(a) consists of an LDL-like particle and the specific apolipoprotein(a) [apo(a)], which is covalently bound to the apoB100 of the LDL like particle through disulphide bridges. Lp(a) causes atherogenesis due to the LDL particle and leads to thrombogenesis due to its structural homology with plasmoinogen, thereby promoting the development of atherosclerotic plaques. Lp(a) also stimulates smooth muscle proliferation of the affected blood vessels. One proposed mechanism by which it is done is by inhibiting the activation of Transforming Growth Factor-β.

Transforming Growth factor type-β (TGF-β) is a superfamily of ligands, receptors binding proteins and ligand traps that together plays a key role in maintaining the normal vessel wall structure by inhibiting smooth muscle proliferation. TGF-β is found to play a crucial role in the development and/or regression of malignant tumors, autoimmune diseases, organ fibrotic changes, kidney diseases and cardiovascular diseases. It exists in three known subtypes in humans TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is present in endothelial cells, vascular smooth muscle cells (VSMCs), myofibroblasts, macrophages, and other hematopoietic cells. It is recognized as the most pivotal TGF-β isoform for the cardiovascular system

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Peripheral Vascular disease, commonly referred to as **peripheral arterial disease** (PAD) or **peripheral artery occlusive disease** (PAOD), refers to the obstruction of large arteries not within the coronary, aortic arch vasculature, or brain. PVD can result from atherosclerosis, inflammatory processes leading to stenosis, an embolism, or thrombus formation[2]. Peripheral arterial disease causes limb pain with exertion, reduces functional capacity and quality of life, and is frequently associated with coronary, cerebral, and renal artery disease . Individuals with PAD are at increased risk for acute cardiovascular events such as myocardial infarction, cerebrovascular attack, aortic aneurysm rupture, and vascular death, as well as ischemic ulceration and amputation . This increased risk for cardiovascular morbidity and mortality is seen even in patients without symptoms[3]. Several population-based studies based on predominantly white European populations have found the prevalence of PAD to be between 6% and 18% over the age of 55 years. The prevalence rises with age and has been found to be approximately 20% in people over 70 years of age and up to 60% in the over 85 age group.

There has, however, been very little research into the prevalence of PAD in non-Caucasian populations, although previous population-based studies have shown variations in the prevalence of this disease amongst different ethnic groups[4]. Peripheral vascular disease affects 1 in 3 diabetics over the age of 50. 70%–80% of affected individuals are asymptomatic; only a minority ever requires revascularisation or amputation.

Classification[5]

Fontaine stages of peripheral arterial disease

- mild pain when walking (claudication), incomplete blood vessel obstruction;
- severe pain when walking relatively short distances (intermittent claudication), pain triggered by walking "after a distance of >150 m in stage IIa and after <150 m in stage IIb"
- pain while resting (rest pain), mostly in the feet, increasing when the limb is raised;
- Biological tissue loss (gangrene) and difficulty walking.

Rutherford classification is a recent classification system and it consists of three grades and six categories:

1. Mild claudication
2. Moderate claudication
3. Severe claudication
4. Ischemic pain at rest
5. Minor tissue loss
6. Major tissue loss

Symptoms[2]

About 20% of patients with mild PAD may be asymptomatic; other symptoms include

- Claudication - pain, weakness, numbness, or cramping in muscles due to decreased blood flow
- Sores, wounds, or ulcers that heal slowly or not at all
- Noticeable change in color (blueness or paleness) or temperature (coolness) when compared to the other limb (termed unilateral dependent rubor; when both limbs are affected this is termed bilateral dependent rubor)
- Diminished hair and nail growth on affected limb and digits.

The incidence of symptomatic PVD increases with age, from about 0.3% per year for men aged 40–55 years to about 1% per year for men aged over 75 years. The prevalence of PVD varies considerably depending on how PAD is defined, and the age of the population being studied. Diagnosis is critical, as people with PAD have a four to five times higher risk of myocardial infarction or stroke.

Diagnosis of PVD[6]

Conventionally PVD is diagnosed by measuring the Ankle Brachial Pressure Index (ABPI) or Ankle Brachial Index (ABI)

Calculation of ABI:

$$\text{ABI} = \frac{\text{Ankle systolic Pressure}}{\text{Highest Brachial systolic pressure}}$$

>/= 1 : normal

0.5 to 1 : moderate disease

< 0.5 : severe disease

<0.3 : critical limb ischemia

PAOD is diagnosed when the ABI is <0.9.

Other techniques used for diagnosis are[2]

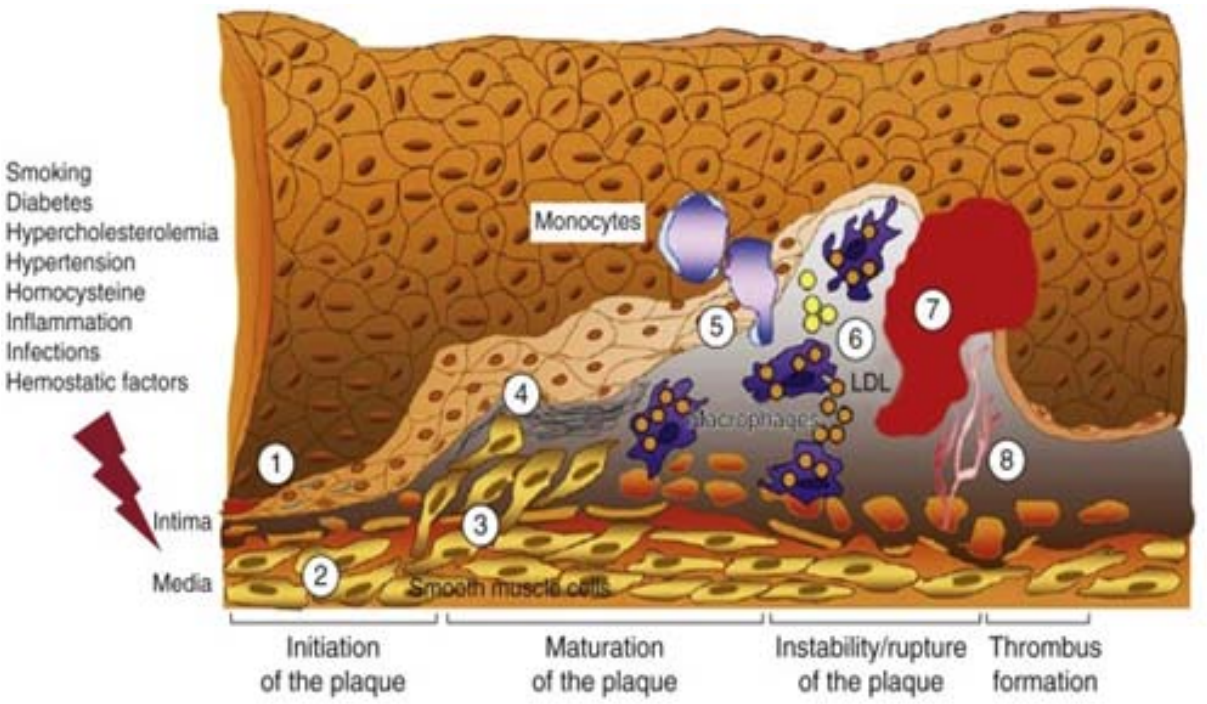
- Doppler ultrasound imaging
- Angiography
- Multislice computerised tomography (CT) scan.

Causes of PVD

ATHEROSCLEROSIS

Atherosclerosis accounts for most peripheral arterial occlusive disease. Many of the risk factors for atherosclerotic coronary artery disease (CAD) such as hyperlipidemia have been identified as risk factors for peripheral arterial disease. Atherosclerosis is a complex disease in which numerous diverse etiologic factors play a role. The most widely accepted concept of genesis of atherosclerosis is that intimal injury incites a series of reactions, which ultimately culminate in development of fibrous plaques. This is the "response to injury" hypothesis[6]. **(fig 1)**

Fig 1 : Evolution of arterial wall changes and plaque formation in the response-to-injury hypothesis

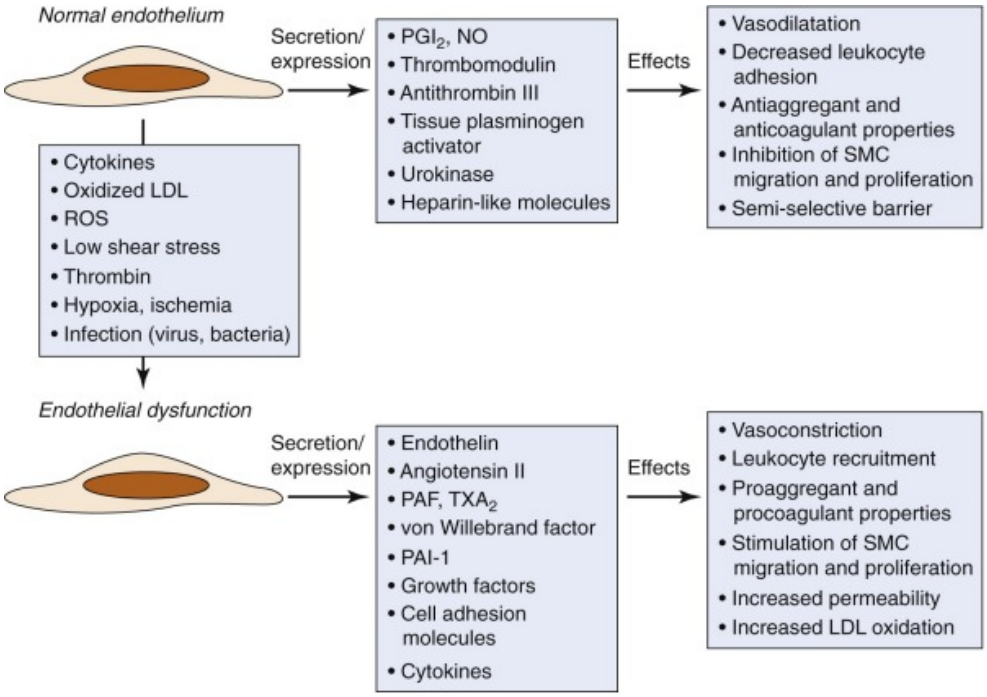


1. Endothelial dysfunction; 2,; 3,; 4,; 5,; 6,; 7,; 8,
2. vascular smooth muscle cell (VSMC) hypertrophy
3. migration and proliferation of VSMCs
4. matrix elaboration
5. expression of adhesion molecules and migration of monocytes
6. uptake of low-density lipoprotein (LDL) and formation of foam cells
7. thrombus formation
8. angiogenesis and neovascularization

Atherosclerosis is defined as an intimal disease of large and medium sized arteries of external diameter more than 2 mm[7]. There is focal accumulation of lipid and smooth muscle cell proliferation producing lesions called plaques which begin mostly in the 2nd or 3rd decade of life[8]. Every plaque has 2 major constituents namely, the lipid and the extra cellular matrix proteins. Collagen produced by the smooth muscle cells is the predominant protein here. The nature and composition of these plaques change as they evolve.

Atherosclerosis reflects a continuing repair process occurring in the arterial wall secondary to persistent arterial injury. The injurious factors could be multifactorial and include hyperlipidemia, shear stress, hypertension, and cigarette smoking. The common denominator is endothelial injury. (**Fig 2)** This results in accumulation of blood-borne monocytes, which migrate into the subendothelial space. Such monocytic accumulation is one of the earliest detectable precursors in the genesis of atherosclerotic lesions. Within the subendothelial space the monocytes convert into cholesterol-laden foam cells. This accumulation distorts the endothelial covering, causing microseparation of endothelial cells and platelet deposition. Smooth muscle cells migrate into the intima from the media, also converting into foam cells[7].

Fig 2 : Consequences of Endothelial Dysfunction



Normal endothelium displays antiaggregant, anticoagulant, and vasodilatative properties, along with inhibition of cell proliferation. After exposure to various agents causing endothelial dysfunction, these functions are modified toward procoagulant and vasoconstrictive activities together with stimulation of cell recruitment and proliferation.

LDL- low-density lipoprotein

NO- nitric oxide

PAF- platelet-activating factor

PAI-1- plasminogen activator inhibitor-1

PGI_2- prostacyclin

ROS- reactive oxygen species

SMC-smooth muscle cell

TXA_2, thromboxane A_2.

T lymphocytes recruited to the intima interact with macrophages and can generate a chronic immune inflammatory state. The T lymphocytes found in atherosclerotic lesions are polyclonal, which indicates that these cells do not develop in response to a single antigen[9]. Activation of complement seems to play a role in both initiation of atherosclerosis and acceleration of the disease. Complement activation can occur by either the classical (antibody dependent) or the alternative (antibody independent) pathway. Cholesterol particles have been shown to be potent complement activators. Proliferation plus activation of VSMCs and endothelial cells is also mediated by activation of complement[10].

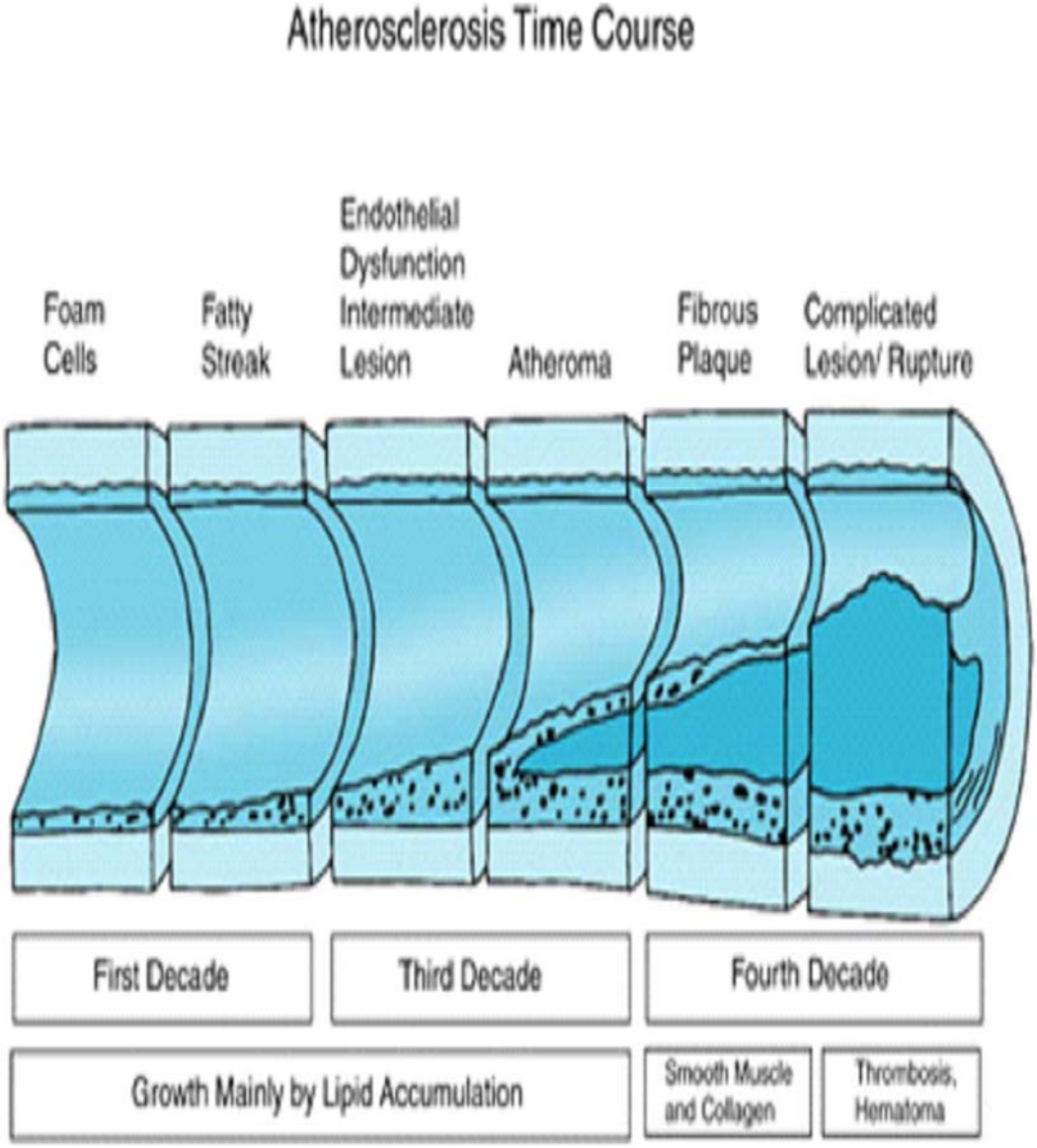
Platelets play an important role in stimulating the progression of atherosclerotic lesions by secreting growth factors and vasoactive substances (e.g., platelet-derived growth factor [PDGF], transforming growth factor-α [TGF-α], TGF-β, epidermal growth factor [EGF], and insulin-like growth factor-I [IGF-I]) after their adherence to the vessel wall in sites of endothelial ulceration[11]. Recently, platelets have been suggested as initial role players in the development of atherosclerotic lesions by recruiting and binding to leukocytes, endothelial cells, and circulating progenitor cells and initiating transformation of monocytes into macrophages. Platelets internalize oxidized phospholipids, express various scavenger receptors that are able to regulate LDL uptake, and promote foam cell formation[12].

The fatty streak is the earliest identifiable lesion of atherosclerosis. It has been detected in children as young as 10 years of age and consists of lipid-laden macrophages overlying

lipid-laden smooth muscle cells. They occur at the same anatomic sites as subsequent fibrous plaques[6].

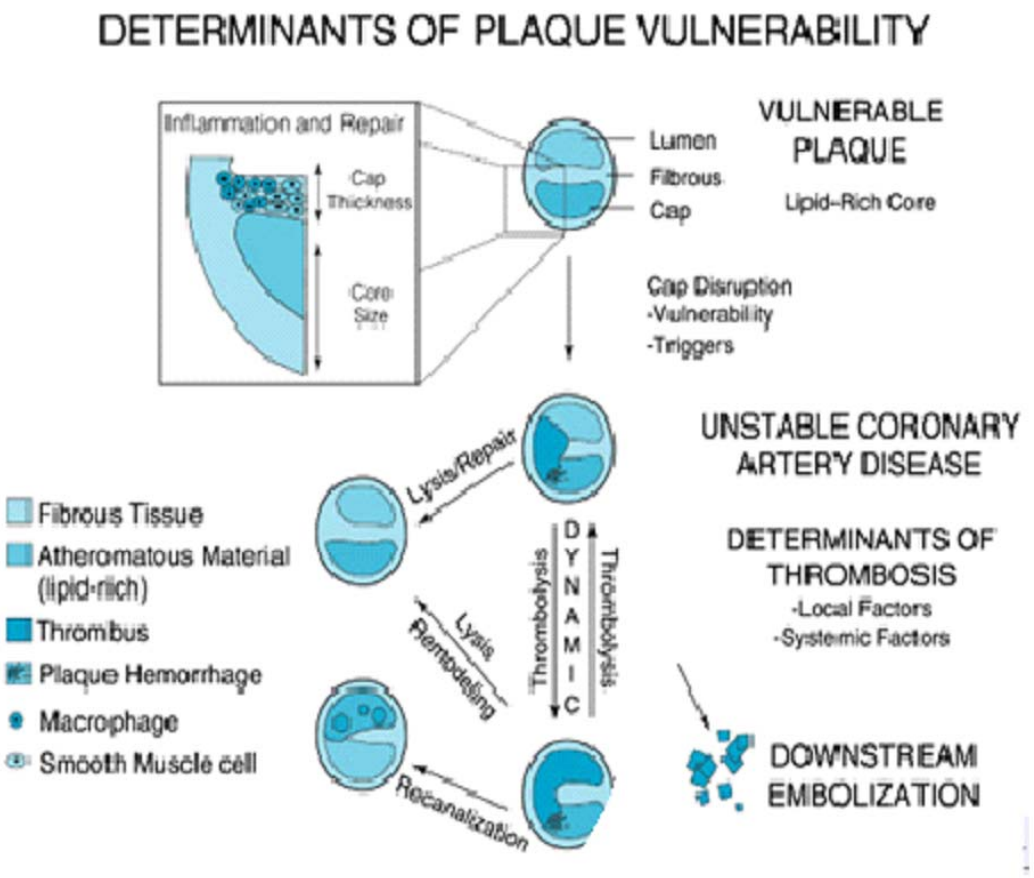
As the lesion grows, as shown in (**fig 3)** it encroaches into the lumen of the vessel which can occlude the vessel. The plaque can also erode into the media or rupture or fissure which processes can allow blood to enter and disrupt the arterial wall or precipitate thrombosis or local vasospasm[13].

Fig 3 : Atherosclerosis Time course



A Vulnerable atherosclerotic plaque (high-risk or unstable plaque) (**fig 4)** is associated with an increased risk of disruption, distal embolization, and vascular events. Vulnerable plaque is an advanced histologic lesion with a large lipid core (filled with lipid and cell debris), a thin fibrous cap, ulceration, intraluminal thrombosis, and intraplaque hemorrhage, as well as intense infiltration by macrophages and other inflammatory cells[14,15].

Fig 4 : Determinants of plaque vulnerability



Categories of Risk Factors for Atherosclerosis and Cardiovascular Diseases[16-20]

CONVENTIONAL

- smoking
- Diabetes mellitus
- Hyperlipidemia

PREDISPOSING

- Advanced age
- Overweight/obesity
- Physical inactivity
- Gender: male sex, postmenopausal women
- Insulin resistance
- Family history/genetics
- Behavioral/socioeconomic factors—race

CONDITIONAL

- Homocysteine
- C-reactive protein (high-sensitivity CRP)
- Fibrinogen
- Lipoprotein (a)

- Hypertriglyceridemia

EMERGING (NOVEL)

- Inflammatory markers
    - Serum amyloid A (SAA)
    - White blood cell count (WBC)
    - Cytokines (IL-1β, IL-6, IL-10, IL-18, monocyte chemotactic protein-1 [MCP-1]
    - Cell adhesion molecules (ICAM-1, VCAM-1, P-selectin, etc.)
    - Soluble CD40 ligand (sCD40L)
    - Protease-activated receptors (PARs)
    - Erythrocyte sedimentation rate (ESR)
    - Lipoprotein-associated phospholipase A_2 (LP-PLA_2)

- Infectious agents
    - *Chlamydia pneumoniae*,
    - Cytomegalovirus (CMV)
    - Herpes simplex virus (HSV) 1 and 2
    - *Helicobacter pylori*
    - Hepatitis A virus
- Vascular calcification markers
    - Osteopontin (OPN)

    - Osteoprotegerin (OPG)
    - Setuin
- Hemostatic factors and hypercoagulable states
    - Lupus anticoagulant
    - D-dimers
    - Markers of platelet activation
    - Tissue plasminogen activator (tPA)
    - Plasminogen activator inhibitor-1 (PAI-1)
    - Prothrombin 1 and 2
    - Protein Z
- Matrix metalloproteinases
- Adipokines: leptin, adiponectin
- Endothelial progenitor cells
- Creatinine
- Urate
- Microalbuminuria
- Small dense low-density lipoprotein (LDL) (sdLDL)
- Oxidative stress
    - Oxidized LDL (ox-LDL)
    - Lectin-like oxidized LDL receptor-1 (LOX-1)
    - Myeloperoxidase
    - Oxidant capacity
    - Reactive oxygen species (ROS)
- Miscellaneous

- Alcohol
- Pregnancy-associated plasma protein-A (PAPP-A)
- Asymmetric dimethylarginine (ADMA)
- Heat shock proteins (HSPs)

The commoner and more prevalent risk factors are discussed briefly as follows:

Age:

Athereosclerotic lesions increase with age[21]

Sex:

Atherosclerotic diseases have increased prevalence in men than in women. The favorable factors for women include higher high density lipoprotein level throughout their life, lower triglyceride level, less central obesity, protection due to estrogen and lower iron storage levels[22]. Diabetes Mellitus is a strong risk factor among women, nearly eliminating the normal protection offered by estrogen. With menopause, Low Density Lipoprotein level begins to rise, whereas HDL levels stop climbing or decrease slightly. This leads to worsening of LDL:HDL ratio. Estrogen may have direct atheroprotective effects on the vessel wall through estrogen receptors[23].

Family History:

Atherosclerosis and CHD are found to run in families. It may be due to the influence of genetic factors as hyperlipidemia and hyperfibrinogenemia are said to be genetically

determined. It has also been attributed to the shared family environment, particularly dietary and social habits[21].

Sedentary life style:

Physical inactivity increases the risk of atherosclerosis. Regular Physical exercise is found to have a protective effect against atherosclerosis by increasing HDL, lowering blood pressure, reducing obesity, reducing blood clotting, promoting collateral vessel development and improving insulin sensitivity[24].

Diet:

Diet deficient in poly unsaturated fatty acids are associated with increased risk of atherosclerosis. Low levels of vitamin C, vit E and other antioxidants may enhance the production of oxidized LDL involved in the pathogenesis of atherosclerosis[21].

Smoking:

Tobacco smoking continues to have a devastating impact on public health and is a critical modifiable risk factor for atherosclerosis, including peripheral arterial disease (PAD). Cigarette smoking mediates its adverse cardiovascular effects through deleterious effects on the artery wall, particularly the endothelium, along with effects on sympathetic tone, metabolism, and the coagulation and fibrinolytic systems. The central components that lead to these adverse effects are carbon monoxide and nicotine[25-29]

Mechanisms of Adverse Cardiovascular Effects of Cigarette Smoking

VASCULAR INJURY

- Endothelial cell damage
- Increased platelet and leukocyte adhesion to endothelial cells
- Impaired nitric oxide bioavailability causing abnormal vasomotor tone
- Increased endothelin-1, a potent vasoconstrictor

METABOLIC ABNORMALITIES

- Increased total and LDL cholesterol
- Increased triglycerides
- Decreased HDL cholesterol
- Insulin resistance

HEMATORHEOLOGIC

- Increased platelet aggregation
- Increased fibrinogen and factor VII
- Increased tissue factor expression
- Impaired release of t-PA- tissue plasminogen activator
- INFLAMMATION
    - Source of oxidative stress

- Increased inflammatory markers (C-reactive protein, WBC count)
- Increased activity of MMPs- matrix metalloproteinase

INCREASED SYMPATHETIC TONE

- Increased heart rate
- Increased blood pressure
- Increased myocardial oxygen demand
- Peripheral vasoconstriction

Smoking cessation rapidly and markedly reduces the risk for coronary atherosclerosis indicating that the responsible processes are reversible to some extent[30].smoking has been shown to be twice as likely to cause PAD as coronary artery disease[31]. The estimated fraction of PAD attributable to smoking is as high as 76%[32]

Alcohol:

It is another prominent risk factor for atherosclerosis in that it affects the lipid profile. Though moderate alcohol intake is found to be associated with higher concentrations of HDL, increased apo A1 and decreased fibrinogen[33], chronic heavy alcoholism is associated with increased triglycerides and increased risk for atherosclerotic complications.

Obesity:

The central distribution of body fat is an independent risk factor for atherosclerosis in spite of the frequent association with other adverse effects such as hypertension, diabetes, and physical inactivity. Obesity has been found to promote insulin resistance, hyperinsulinemia, hypertension, hypertriglyceridemia, and increased LDL cholesterol involved in the pathology of atherosclerosis[9,24].

DIABETES MELLITUS

DM is a strong independent risk factor for atherosclerosis increasing risk by atleast 2 times in both sexes. The proatherogenic changes associated with diabetes may predate its diagnosis and include derangements in the regulation of blood flow, abnormalities in the components of blood vessels, and alterations in coagulation and rheology. In addition to increasing the burden of disease, these derangements result in the activation of inflammatory pathways, which increases the activity of the disease. These changes are associated with an increased risk for accelerated atherogenesis, as well as poor outcomes[34].

Endothelial cell dysfunction is key to the pathophysiology of atherosclerosis in DM. Several mechanisms contribute to the endothelial dysfunction, including hyperglycemia, free fatty acid (FFA) production, and most importantly, insulin resistance[35]. Hyperglycemia blocks the function of endothelial nitric oxide synthase (eNOS) and boosts the production of reactive oxygen species, which impairs the vasodilator homeostasis fostered by the endothelium. This oxidative stress is amplified because in

endothelial cells, glucose transport is independent of insulin and not downregulated by hyperglycemia[36].

In addition to hyperglycemia, insulin resistance plays a role in the loss of normal NO homeostasis. NO is a potent stimulus for vasodilatation and limits inflammation via its modulation of leukocyte–vascular wall interaction. Furthermore, NO inhibits vascular smooth muscle cell (VSMC) migration and proliferation and limits platelet activation. Therefore, the loss of normal NO homeostasis can result in risk for a cascade of events in the vasculature that lead to atherosclerosis and its consequent complications[37].

The common precipitating factors of atherosclerosis in DM are increased LDL, decreased HDL, high triglycerides that in turn increases triglyceride rich lipoprotein remnant particles. Increased small dense LDL, elevated lipoprotein(a), enhanced lipoprotein oxidation, glycation of LDL, increased fibrinogen, increased platelet aggregability, impaired fibrinolysis, plasminogen activator inhibitor-1 (PAI-1)[38].

DYSLIPIDEMIA

The essential role of atherogenic lipoproteins in the pathogenesis of atherosclerotic vascular disease has been well established, as well as the benefits of lipid management for the primary prevention and amelioration of existing atherosclerotic vascular disease. Although much of the evidence on the management of lipid disorders has resulted from studies on atherosclerotic cardiovascular disease and, to a lesser extent, cerebrovascular disease, this experience is applicable to the prevention and treatment of peripheral artery

disease (PAD) because of the common pathophysiology of atherosclerosis in any vascular bed[1].

The major lipids in the body are triglycerides, free cholesterol, cholesterol esters and phospholipids.

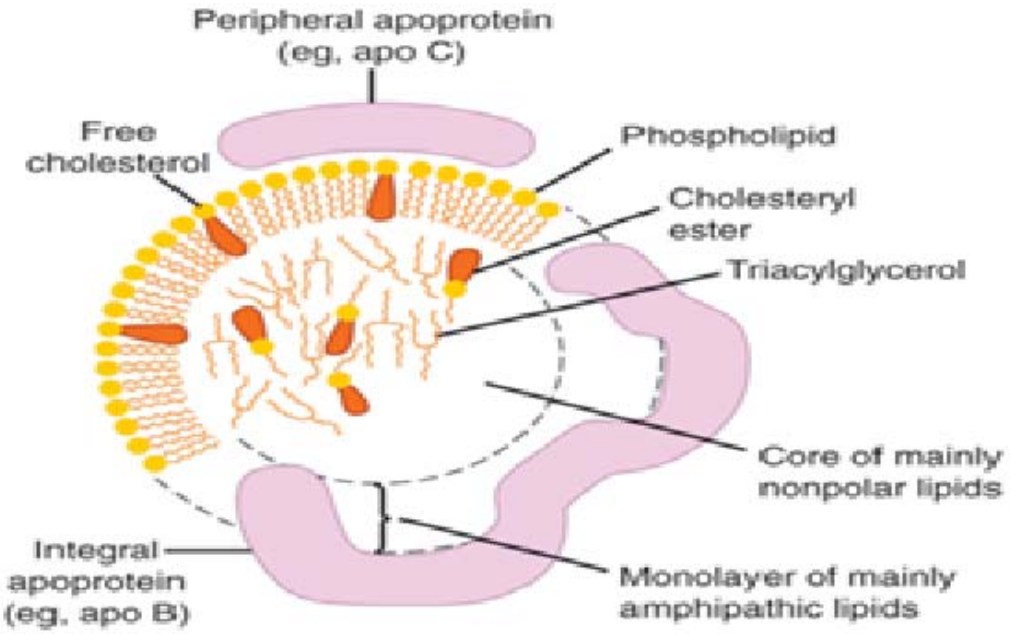
Triglycerides are the storage form of fat; they serve as a source of energy and are stored in the adipose tissue. Cholesterol serves as a component of cell membranes and as a precursor for steroid hormones and bile acids. Phospholipids are the major components of cell membranes and lipid transporting lipoproteins. They are amphipathic lipids.Cholesterol and triglycerides are hydrophobic compounds and cannot dissolve in plasma directly, so they are carried in circulation as a complex with the amphipathic phospholipids and water soluble lipoproteins[39].

LIPOPROTEINS

Lipids are chemically insoluble in the aqueous medium of blood and must be carried by spherical particles in which the hydrophobic lipid components are surrounded by an envelope of hydrophilic phospholipids and proteins known as apolipoproteins. These lipoprotein particles have the principal function of transporting lipids from the intestine and liver through the bloodstream to the various cells of the body where they can be stored, used for important synthetic processes, and metabolized to yield energy. The various apolipoproteins, such as apoB, apo A, apo E, and apo C, serve important functions in metabolism of the contained lipid and also are specific to the binding of lipoproteins to specific receptors on the surface of cells throughout the body. All

lipoproteins are organized into a hydrophobic core of neutral lipids (triglycerides and cholesteryl esters) and a hydrophilic surface coat of polar lipids (free cholesterol and phospholipids) and apolipoprotein (**fig 5**)

Fig 5 : GENERAL STRUCTURE OF LIPOPROTEIN



Although lipoprotein particles, differing in their relative lipid and lipoprotein composition, size, density and function, they actually form a heterogenous continuum, a traditional classification based on the density at which lipoproteins float during ultra centrifugation, divides them into the following classes[40]:

- Chylomicrons
- Very Low Density Lipoproteins
- Intermediate Density Lipoproteins
- Low Density Lipoproteins
- High Density Lipoproteins

Additionally, lipoproteins can be classified on the basis of electrophoretic mobility. In addition to the five lipoprotein classes, a heterogenous class of low density lipoprotein like lipoprotein particles termed Lipoprotein(a), containing apolipoprotein(a) and apolipoprotein B100 as protein moiety has been characterized. Within the classical lipoprotein fractions high density lipoprotein fraction has been shown to be comprised of several distinct subclasses, differing in their density, particle size or apolipoprotein composition[41].

The major characteristics of the lipoproteins have been described in the **table I.**

Table I: Characteristics of the major plasma Lipoproteins

				Composition			
Lipoprotein	**Source**	**Diameter (nm)**	**Density (g/mL)**	**Protein (%)**	**Lipid (%)**	**Main Lipid Components**	**Apolipoproteins**
Chylomicrons	Intestine	90–1000	< 0.95	1–2	98–99	Triacylglycerol	A-I, A-II, A-IV,[1] B-48, C-I, C-II, C-III, E
Chylomicron remnants	Chylomicrons	45–150	< 1.006	6–8	92–94	Triacylglycerol, phospholipids, cholesterol	B-48, E
VLDL	Liver (intestine)	30–90	0.95–1.006	7–10	90–93	Triacylglycerol	B-100, C-I, C-II, C-III
IDL	VLDL	25–35	1.006–1.019	11	89	Triacylglycerol, cholesterol	B-100, E
LDL	VLDL	20–25	1.019–1.063	21	79	Cholesterol	B-100
HDL	Liver, intestine, VLDL, chylomicrons					Phospholipids, cholesterol	A-I, A-II, A-IV, C-I, C-II, C-III, D,[2] E
HDL[1]		20–25	1.019–1.063	32	68		
HDL[3]		10–20	1.063–1.125	33	67		
HDL[3]		5–10	1.125–1.210	57	43		
Pre-HDL[3]		< 5	> 1.210				A-I
Albumin/free fatty acids	Adipose tissue		> 1.281	99	1	Free fatty acids	

Importance of Non–High-Density Lipoprotein Cholesterol*

- Known predictor of CHD in epidemiology
- Equivalent to total apo B-100 and TC/HDL
- Represents the sum of LDL, Lp(a), IDL, and VLDL: all atherogenic apo B–containing lipoproteins
- Lipid equivalent of hemoglobin A_{1c}

CHD, coronary heart disease; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; Lp(a), lipoprotein (a); TC, total cholesterol; VLDL, very-low-density lipoprotein.

* Non–HDL cholesterol = TC − HDL cholesterol.

Apolipoproteins :

Apolipoproteins are specific protein components of lipoproteins. They carry out several roles and their distribution characterizes the lipoprotein. Measurement of these apolipoproteins is representative of the lipid content of the body. They maintain the structural integrity of the lipoprotein complex. They are responsible for the activation of enzymes known to be important in lipid metabolism. They facilitate uptake of lipoproteins by cell specific surface receptors.

Classification and properties of major plasma lipoproteins are given in the **table II.**

Table II : Classification and Properties of major plasma Apolipoproteins

Apoliporotein	Molecular weight	Chromosomal location	Function	Lipoprotein carrier
Apo A-I	29016	11	Activates LCAT	HDL
Apo A-II	17414	1	Inhibits LPL	Chylomicron HDL
Apo B-100	512713	2	Secretion of triglyceride from liver binding protein to LDL receptor	VLDL, IDL, LDL
Apo B-48	240800	2	Secretion of triglyceride from intestine	Chylomicron
Apo C-I	6630	19	Activates LCAT	Chylomicron, VLDL, HDL
Apo C-II	8900	19	Cofactor LPL	Chylomicron, VLDL, HDL
Apo C-III	8800	11	Inhibits apo C-II Activation of LPL	Chylomicron, VLDL, HDL
Apo E	31435	19	Facilitates uptake of chylomicron remnant and IDL	Chylomicron, VLDL, HDL
Apo (a)	187000- 662000	6	Thrombogenicity	Lp(a)

The general characteristics of the apolipoproteins central to the pathogenesis of atherosclerosis are discussed here:

Apolipoprotein A-I

- Major protein component of HDL particles (Good Cholesterol)
- Chylomicrons secreted from the intestinal enterocyte also contain ApoA1 but it is quickly transferred to HDL in the bloodstream.
- The protein promotes cholesterol efflux from tissues to the liver for excretion
- ApoA-I was also isolated as a prostacyclin (PGI2) stabilizing factor, and thus may have an anticlotting effect[42]
- It is a cofactor for Lecithin Cholesterol Acyl Transferase (LCAT) enzyme
- **ApoA-1 Milano** is a naturally occurring mutant of ApoA-I. Paradoxically, carriers of this mutation have very low HDL cholesterol levels, but no increase in the risk of heart disease[43].
- ApoA-I binds to lipopolysaccharide or endotoxin, and has a major role in the anti-endotoxin function of HDL[44]
- Defects in the gene encoding it are associated with HDL deficiencies, including Tangier disease, and with systemic non-neuropathic amyloidosis**.**

Apolipoprotein B-100 :

It is a monomeric glycoprotein of molecular weight 550 kDa. It is the most abundant plasma apolipoprotein and the only protein of LDL (Bad cholesterol)[45]

The apo B protein occurs in the plasma in 2 main isoforms, APOB48 and APOB100. The first is synthesized exclusively by the small intestine, the second by the liver. Both isoforms are coded by *APOB* and by a single mRNA transcript larger than 16 kb. APOB48 is generated when a stop codon (UAA) at residue 2153 is created by RNA editing. There appears to be a *trans*-acting tissue-specific splicing gene that determines which isoform is ultimately produced. Alternatively, there is some evidence that a *cis*-acting element several thousand bp upstream determines which isoform is produced.As a result of the RNA editing, APOB48 and APOB100 share a common N-terminal sequence, but APOB48 lacks APOB100's C-terminal LDL receptor binding region. Apo B48 is the chief apoprotein of chylomicrons.

It consists of 4536 amino acid residues and is organized into several domains viz[46].

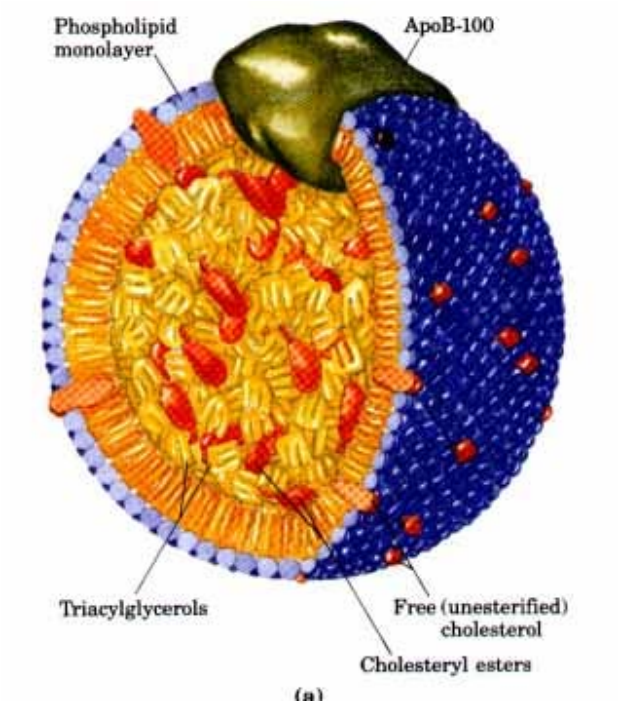
- Lipid binding domain
- Receptor binding domain
- Domains involved in lipoprotein(a) assembly
- Lipoprotein lipase binding domain
- Hepatic lipase binding domain
- Microsomal triglycerides transfer protein- binding domain

At the early stage of production of the apo B molecule, targeting of secretory proteins to the endoplasmic reticulum (ER) is achieved by the translation of the signal sequence and building of this to a signal recognition particle which recognizes a “docking Protein” on the ER. The signal peptide is then cleaved by a protease. So this part of this peptide doesnot appear in the mature circulating protein but may have an important effect on its

rate of transport. Thus the translocation rate of the protein across to the ER is highly likely to be affected by the polymorphism of the gene encoding it.

Apo B-100 I essential for the assembly and secretion of VLDL, maintenance of structural integrity of LDL and for the uptake of LDL by the hepatic receptors[47]. Structure of LDL with apo B100 is given in **fig 6**.

Fig 6 : STRUCTURE OF LDL



The structure of apo B has been analyzed in terms of lipid binding, lipoprotein assembly, and LDL receptor pathway mediated LDL clearance. In apo B 100 few of the predicted alpha helices are truly amphipathic, so are the beta strands that contain alternate hydrophobic and hydrophilic amino acids. Lipid binding structures in the form of amphipathic alpha helices and beta strands and hydrophobic domains are distributed throughout the length of apo B-100 giving the molecule its characteristics of insolubility in aqueous media and non-exchangeability.

The apo B-100 remains tightly attached to its core lipid throughout the molecule transition that leads to the formation of LDL, during which triglyceride and phospholipid are distributed to muscle and adipose tissue and all other VLDL apoproteins like apo E & C are lost to different lipoprotein fractions.

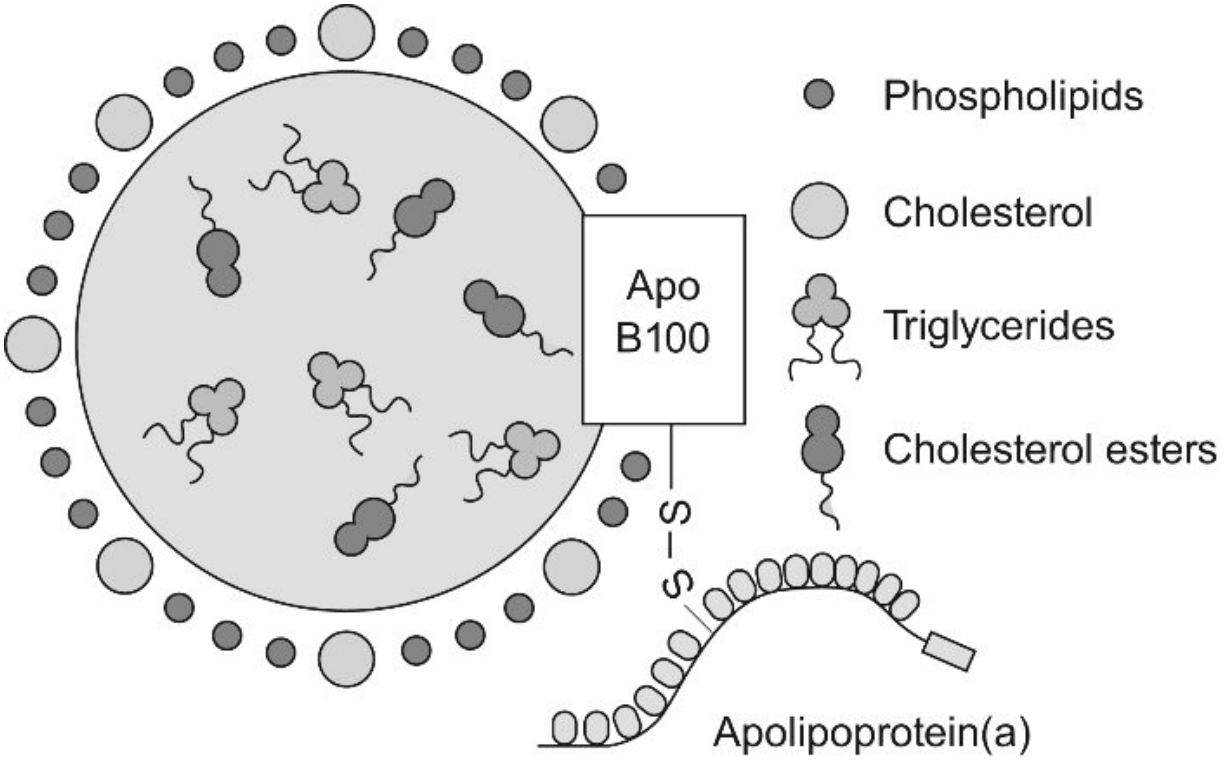
Several features have been identified that may have importance in lipoprotein assembly. Cysteine residues determine the protein tertiary structure. Six out of seven cysteines in apo B-100 are involved in intramolecular disulphide linkage. The cysteine rich domain of apo B-100 may confer specific globular structure that is necessary for nascent lipoprotein assembly and transport form the ER to the Golgi apparatus.

Apolipoprotein(a) :

Apolipoprotein[a] is the highly glycosylated, hydrophilic apoprotein of lipoprotein[a] (Lp[a]). It is covalently bound to the apoB of the LDL like particle.(**fig 7)** Lp(a) plasma concentrations are highly heritable and mainly controlled by the apolipoprotein(a) gene [LPA] located on chromosome 6q26-27. Apo(a) proteins vary in size due to a size

polymorphism [KIV-2 VNTR], which is caused by a variable number of so called kringle IV repeats in the LPA gene. This size variation at the gene level is expressed on the protein level as well, resulting in apo(a) proteins with 10 to > 50 kringle IV repeats (each of the variable kringle IV consists of 114 amino acids)[48].

Fig 7 : STRUCTURE OF Apo(a) IN LIPOPROTEIN (a)



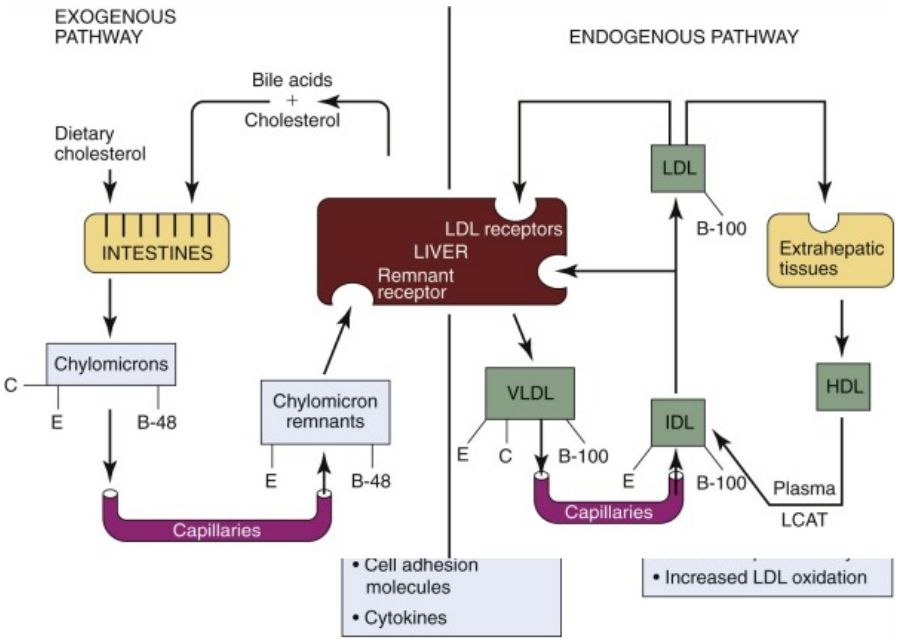
It is generally considered to be a multimeric homologue of plasminogen (kringle domain and a serine protease domain), and to exhibit atherogenic/thrombogenic properties. The kringle domain encompasses 11 distinct types of repeating units, 9 of which contain 114 residues. These units, called kringles, are similar but not identical to each other or to PGK4. Apo[a] kringles are linked by serine/threonine- and proline-rich stretches similar to regions in immunoglobulins, adhesion molecules, glycoprotein Ib-alpha subunit, and kininogen[49].

The pathogenic consequences of this structural peculiarity of apo(a) are described in detail under Lipoprotein(a).

Lipoprotein metabolism

This can be divided into an exogenous and an endogenous phase[50] . **FIG 8.**

FIG 8 : LIPOPROTEIN METABOLISM- EXOGENOUS AND ENDOGENOUS PHASES



HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase, LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein

Chylomicrons :

During the exogenous phase, dietary lipids and lipids that are recirculated in bile are absorbed into enterocytes and packaged as very large lipoproteins called chylomicrons.

Although dietary cholesterol and triglycerides are absorbed by different mechanisms within the gastrointestinal (GI) tract, they are combined in this single chylomicron particle for transport from the GI tract to the rest of the body, with triglycerides constituting approximately 90% of the chylomicron's lipid content. These large lipoproteins are transported from the gut through the thoracic duct and into the bloodstream. Because of their very large size, when in significant concentration, chylomicrons account for the turbidity or “milkiness” of plasma, known as postprandial lipemia, seen in some individuals with a variety of metabolic and inherited disorders.

Chylomicrons are distinguished by the presence of one apo B-48 molecule in each particle. In the bloodstream, the enzyme lipoprotein lipase (LPL) hydrolyzes the triglyceride contained within the chylomicron into free fatty acids, which are then stored in adipocytes and muscle cells to be metabolized for future energy production. The remaining triglyceride-poor chylomicron remnant contains only the absorbed dietary cholesterol, which is then transported to the liver for storage. Recent studies of atherosclerotic lesions have found apo B-48 within plaque, thus implicating these chylomicron remnants as atherogenic particles.

Very-Low-Density Lipoproteins

The endogenous phase of lipoprotein metabolism involves the formation in the liver of very-low-density lipoproteins (VLDLs) containing both cholesterol and triglycerides derived from stores of these two lipids within the liver and adipocytes (Table 28-1). Each VLDL particle contains apolipoproteins from the C and E family and one molecule of apo B-100 per particle. As with chylomicrons, the predominant lipid component of VLDL is triglyceride, which accounts for approximately 70% of its lipid content. Though not as large as chylomicrons, VLDL is large enough to cause lipemia when present in very high concentration. VLDL is released from the liver into the bloodstream, where LPL again facilitates removal of the triglyceride component of VLDL and presents it to the muscle cell as fuel for energy production. Through this mechanism, stored triglycerides are provided to the muscle and other cells during fasting or starvation as a more energy-rich alternative to glucose. As the triglyceride is removed, two additional atherogenic lipoprotein particles are formed, VLDL remnants and intermediate-density lipoproteins (IDLs). These triglyceride-rich lipoprotein particles are atherogenic and play an important role in the accelerated atherosclerosis observed in metabolic syndrome and type 2 diabetes mellitus.

Low-Density Lipoprotein

In addition to LPL, another lipase known as hepatic lipase participates in the conversion of VLDL to LDL, the most atherogenic of all lipoprotein particles. Although a number of other apolipoproteins were attached to the original VLDL particle, only one apo B-100 is present in each LDL particle.

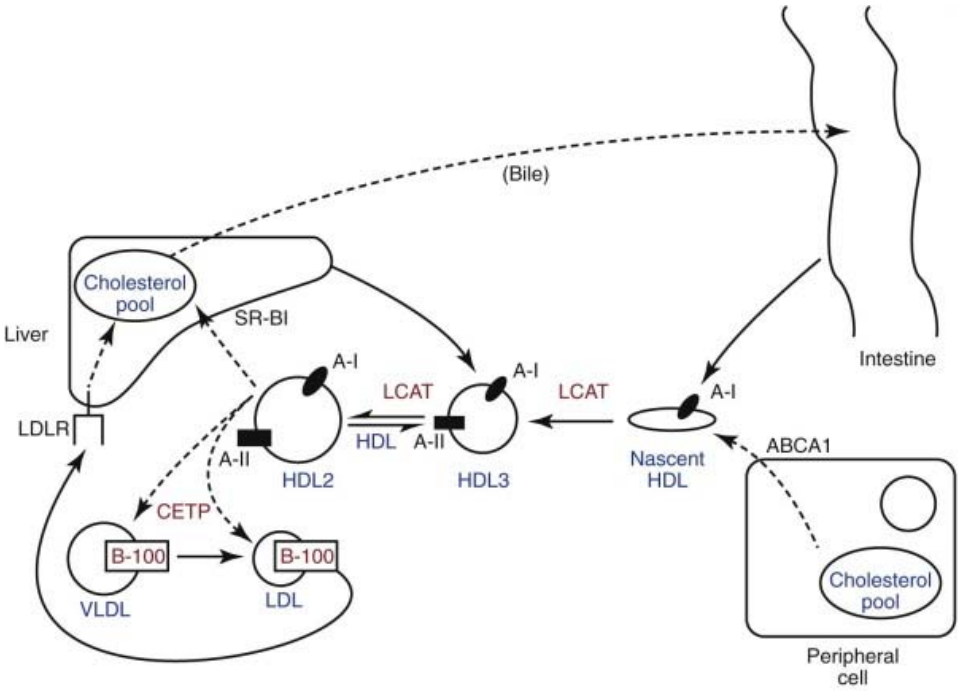
LDL binds to specific LDL receptors on the surface of each cell, and such binding facilitates transfer of the remaining cholesterol to these cells, where it can be stored for future use to make such chemical products as cell membranes, steroid hormones, and bile acids. The circulating LDL concentration in plasma is determined by the number of LDL receptors on the various cells of the body, with the liver accounting for more than 70% of this total receptor number. In turn, the number of LDL receptors is regulated by the intracellular concentration of cholesterol within each cell. When the intracellular cholesterol content of the cells is low, LDL receptor synthesis is upregulated, receptor numbers increase, and the LDL concentration of circulating plasma diminishes. On the other hand, when intracellular cholesterol is increased, LDL receptor synthesis is downregulated, receptor numbers diminish, and LDL within the circulation rises. When plasma LDL is present in excess, atherosclerosis results in proportion to the degree of circulating LDL. Humans are born with a maximum number of LDL receptors and a very low circulating LDL level of 25 to 30 mg/dL (0.65 to 0.78 mmol/L). Over our lifetime, the current lifestyle of excessive calorie, cholesterol, and saturated fat intake and inactivity results in an increasing intracellular cholesterol level, downregulation of LDL receptors, and attainment of the currently observed excess LDL cholesterol levels that has resulted in the epidemic of atherosclerosis seen throughout the world.

High-Density Lipoproteins

HDL is synthesized by the liver and intestine as apo A-I, which is then released into the bloodstream as a lipid-poor discoid particle. As it circulates, stored cholesterol is released from peripheral cells through the action of a specific transporter known as ABCA1

cassette transporter. As cholesterol is absorbed by the discoid apo A-I and converted to cholesterol ester under the influence of lecithin-cholesterol acyltransferase (LCAT), HDL becomes a spherical particle. Additional cellular cholesterol is then added by another cassette transporter, ABCG1, and through the action of the receptor SR-B1. The HDL particle can then return to the liver, where it binds to hepatic SR-B1 and releases its cholesterol, or it can exchange a portion of its cholesterol content for triglyceride from VLDL through the chemical action of the cholesterol ester transfer protein (CETP). The exchanged cholesterol can then be transported back to the liver by LDL. This process is known as "reverse cholesterol transport" and plays an important role in the antiatherogenic properties of the HDL particle[51]. **FIG 9.**

FIG 9 : REVERSE CHOLESTEROL TRANSPORT OF HDL



ABCA1, adenosine-binding cassette transporter 1; CETP, cholesterol ester transfer protein; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, LDL receptor; SR-B1, scavenger receptor type B1; VLDL, very-low-density lipoprotein

Table III : ATP III Guidelines for the Evaluation of Fasting Lipid Profile

	Conventional Units(mg/dL)	SI units (mmol/L)
Low-Density Lipoprotein Cholesterol		
Optimal	<100	<2.59
Near optimal	100-129	2.59-3.34
Borderline high	130-159	3.37-4.12
High	160-189	4.14-4.90
Very high	>190	>4.92
High-Density Lipoprotein Cholesterol		
Low	<40	<1.04
High	>60	>1.55
Triglycerides		
Normal	<150	<1.70
Borderline	150-199	1.70-2.25
High	200-499	2.26-5.64
Very high	>500	>5.65

Hence the endogenous lipid transport system can be divided into two subsystems:

- Apo A-I lipoprotein system (high density lipoprotein)
- Apo B-100 lipoprotein system (VLDL, IDL, LDL)

Apo A-I lipoprotein system:

HDL particles are the main mediators of the reverse cholesterol transport system whereby cholesterol synthesized or deposited in peripheral cells is returned to liver[52].

This process begins with the removal of free cholesterol from the cel membranes to nascent HDL particles secreted by the liver and intestine, and esterification of free cholesterol by lecithin cholesterol acyl transferase after which the cholesteryl ester I stransferred to the hydrophobic core of the HDL particle[53]. In this process nascent HDL is converted to spherical lipid- rich HDL. Part of the HDL core cholesteryl ester is then transferred to apolipoprotein B-48 or apolipoprotein B-100 containing lipoproteins in exchange for triglycerides by the cholesteryl ester transfer protein (CETP). The cholesteryl esters remaining in the HDL particles are taken up by the hepatocytes either via receptor- mediated endocytosis of apolipoprotein E containing HDL particles by the remnant receptor or through selective removal of HDL cholesteryl ester by the hepatic HDL receptor. At the same time triglycerides transferred from the other lipoproteins to HDL are hydrolyzed by hepatic lipase, leading to the conversion of triglycerides – rich HDL_2 to triglycerides poor HDL_3 particles, and the release of free apolipoprotein A-1 and lipid poor HDL to be reused in the reverse cholesterol transport cycle. Besides the exchange of cholesteryl ester for triglycerides, the complex interplay of HDL with other lipoproteins during reverse cholesterol transport involves exchange of other components as well, such as apolipoproteins and phospholipids[54].

Apo B-100 lipoprotein system:

The apolipoprotein B-100 system begins with the hepatic assembly and secretion of apo B-100 containing VLDL particles. Therafter VLDL- triglycerides are hydrolyzed in peripheral tissues by lipoprotein lipase (LPL) and the particles are converted to smaller triglyceride depleted intermediate density lipoprotein (IDL)[55]. The liver via the LDL receptor conceivably removes some of the IDL particles. The rest are converted to LDL particles.

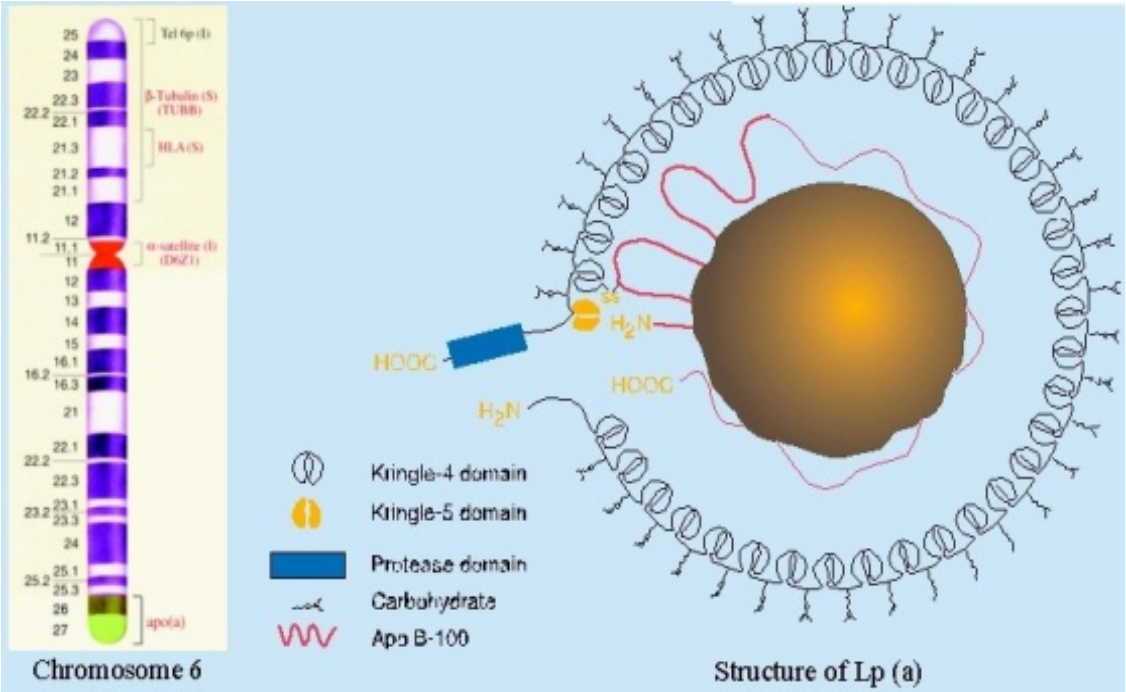
The liver through the LDL receptor clears most of the LDL particles. Other receptors and non-receptor mediated uptake through still poorly defined pathways play a smaller role in LDL clearance[56]. Concentration of LDL considerably varies within population.

Lipoprotein(a):

Lipoprotein Lp(a) is a major and independent genetic risk factor for atherosclerosis and cardiovascular disease. The essential difference between Lp(a) and low density lipoproteins (LDL) is apolipoprotein apo(a), a glycoprotein structurally similar to plasminogen, the precursor of plasmin, the fibrinolytic enzyme[57]. Lp(a), is an LDL-like particle discovered by Berg in 1963.

Lp(a) is a complex particle composed of a lipid core and two disulfide-linked subunits: apolipoprotein B-100 and apolipoprotein apo(a). The lipid core and apo B-100 of Lp(a) are shared with LDL; in contrast, the apo(a) glycoprotein confers its characteristic properties on Lp(a). Apo(a) shows a high degree of homology with plasminogen, the precursor of the fibrinolytic enzyme plasmin. **FIG 10.**

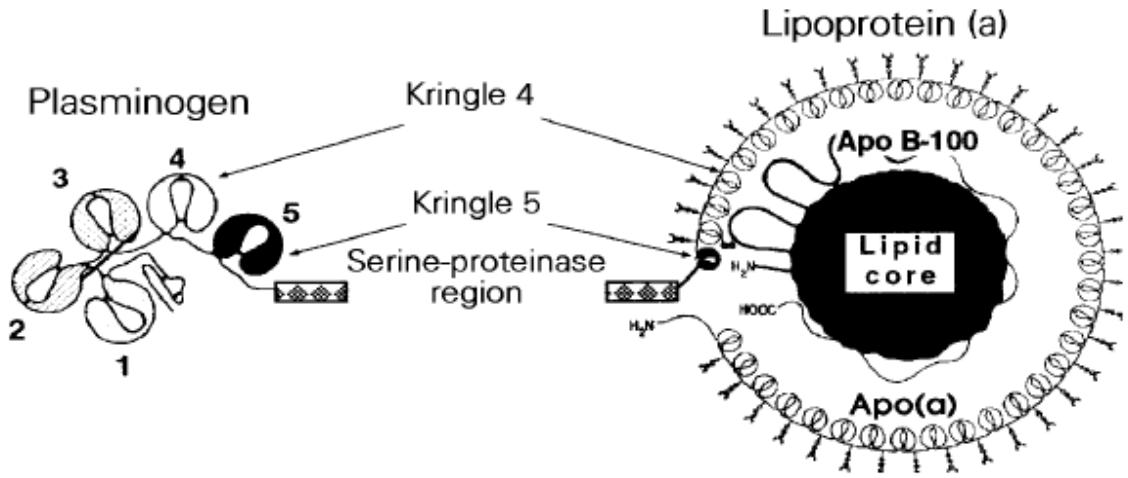
FIG 10 : STRUCTURE OF LIPOPROTEIN(a)



The fact that Lp(a) has both LDL and plasminogen-like moieties suggests that Lp(a) may constitute a link between the processes of atherosclerosis and thrombosis. Lp(a) and fibrin have been identified in atherosclerotic plaques[58].

Plasminogen and apolipoprotein(a) are homologous proteins with opposite effects. **FIG 11.**

FIG 11 : STRUCTURAL HOMOLOGY OF APO(a) WITH PLASMINOGEN



Plasminogen is a single-chain glycoprotein of Mr 93,000 secreted by the liver and found in plasma at a concentration of 1.5 to 2 μmol/l. It consists of 791 amino acid residues arranged in two types of domains with functional autonomy: the kringle modules and the serine-proteinase region. Kringles are sequences of 80-90 amino acids arranged in a triple-loop tertiary structure rigidly stabilized by three disulfide bridges. The kringle structure was first described in prothrombin and is found in several copies in proteins that evolved from a common ancestral gene, i.e., plasminogen, apo(a) and hepatocyte growth factors[59]. The kringle domains of plasminogen, designated 1 to 5, differ from each other and are connected to the proteinase domain by a sequence adjacent to the activation cleavage site Arg561-Val562. The serine-proteinase domain contains the active catalytic center (Ser741, His603, Asp646) and is located in the carboxy-terminal region (Val562-Asn791), whereas the amino-terminal region (Glu1-Arg561) bears the five kringle domains and an amino-terminal peptide of 77 residues (Glu1-Lys77) that may be released by plasmin. Thus, native plasminogen possesses a glutamic acid as the amino-terminal residue (Glu-plasminogen), while the corresponding residue in the plasmin-cleaved form is lysine (Lys-plasminogen). Lys-plasminogen is not normally found in human plasma.

Kringles 1 and 4 of plasminogen contain a functional subsite supported primarily by amino acid residues of the inner loop. Since this subsite binds to lysine residues of fibrin and cell membrane proteins it has been termed lysine-binding site or LBS. The structure of this subsite, an ionic dipole with the anionic and cationic sites positioned at opposite ends of a hydrophobic trough, has been well defined. In both kringle 1 and kringle 4, the anionic center is constituted by Asp55 and Asp57 while the cationic center is mainly represented by Arg34 and Arg71 in kringle 1, and by Lys35 and Arg71 in kringle 4[60].

The specific interactions between lysine residues in fibrin or cell membrane proteins and the lysine-binding subsites in kringles 1 and 4 of plasminogen allow plasminogen binding and activation.

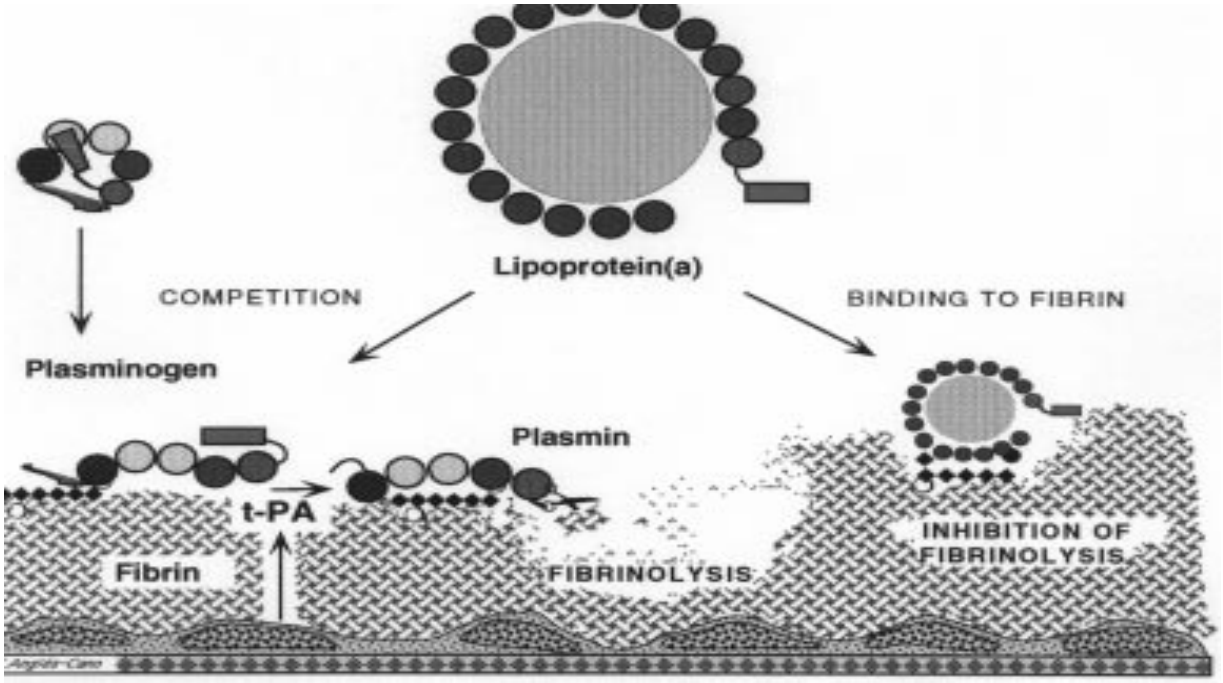
Apo(a) contains a variable number of kringle domains that share 61-75% homology with kringle 4 of plasminogen. The kringle 4-like repeats of apo(a) are followed by a single copy of plasminogen kringle 5 and a protease domain that shares 94% homology with the corresponding domain of plasminogen[61]. Kringle 4 copies of plasminogen in apo(a) are similar but not identical and have been classified into 10 different types. Kringle 4 type 2 presents the lowest degree of homology with plasminogen kringle 4 and has no functional LBS; the number of this type of kringle in apo(a) is variable and gives rise to a series of apo(a) isoforms that contribute to the heterogeneity of Lp(a): a total of 34 apo(a) alleles and glycoproteins with molecular masses ranging from ~300 to ~800 kDa have been identified by protein (13) and cDNA (14) analysis. The other nine kringle types are present as single copies in all isoforms; kringle 4 type 9 possesses an additional cysteine residue that ensures the covalent binding between apo(a) and apo B-100 and thereby the formation of the Lp(a) particle[62].

Sequence comparison and molecular modeling have shown that a lysine-binding pocket similar to that of plasminogen kringle 4 is present in kringle 4 type 10 of apo(a) and that slightly modified LBS are present in kringle 4 types 5 to 8 these kringle copies may confer binding capabilities similar to those of plasminogen on apo(a)[63]. However, the Arg-Val residues of the activation cleavage site in plasminogen have been replaced by Ser-Ile in apo(a), a substitution that impairs recognition of apo(a) by plasminogen activators.

Thus, binding of apo(a) instead of plasminogen to fibrin and cell surfaces may result in a diametrically opposed effect, i.e.,inhibition of the generation of plasmin.

Inhibition of the generation of plasmin is the major mechanism of action of Lp(a). **FIG 12**

FIG 12 : LIPOPROTEIN(a) INHIBITS FIBRINOLYSIS

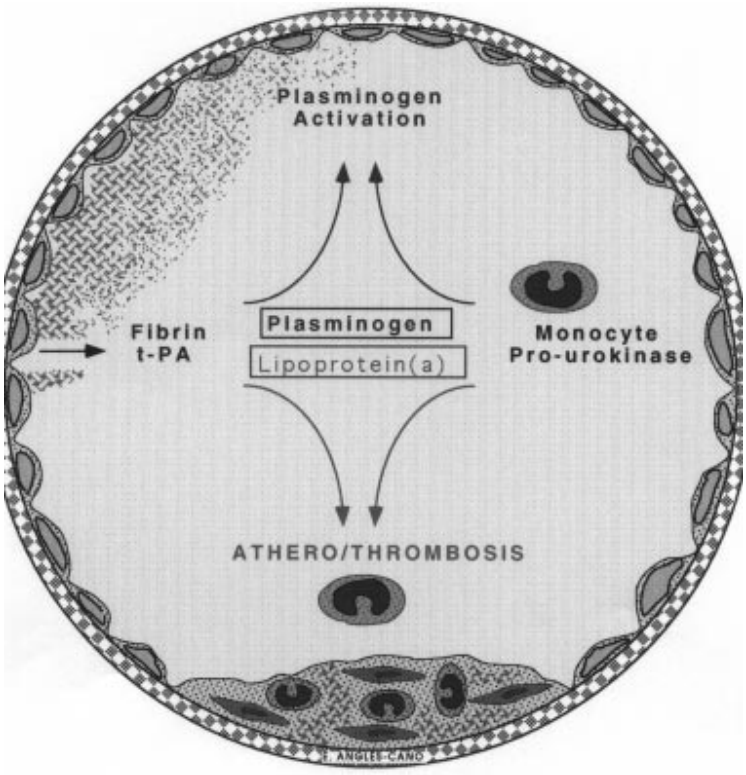


Initial limited degradation of the surface of fibrin by plasmin unveils carboxy-terminal lysine residues and increases the local concentration of plasminogen, a process that amplifies and accelerates the degradation of fibrin. In a plasma milieu, the progression of such a process is markedly influenced by a2-antiplasmin, the specific plasmin inhibitor, which limits the number of carboxy-terminal lysine residues and thereby the amount of bound plasminogen[64]. Since the kringle domains behave as autonomous functional structures, the presence in apo(a) of kringle modules structurally related to those of plasminogen may result in analogous interactions with lysine residues of fibrin and cell membranes.

Thus, Lp(a) interferes with the evolution of fibrinolysis on the surface of fibrin, endothelial cells, monocytes and platelets through binding of apo(a), an eternal zymogen that decreases the local concentration of plasminogen and cannot be transformed into an active enzyme[65].

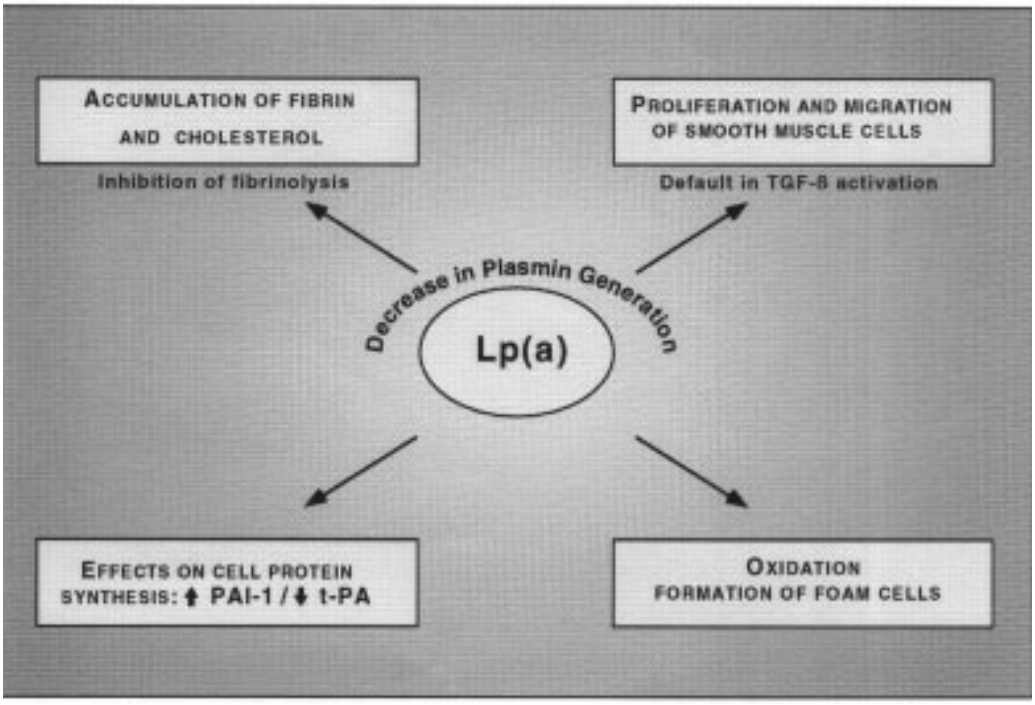
Effects of Lp(a) such as persistence of fibrin deposits, accumulation of cholesterol and proliferation of smooth muscle cells in the intima are related to a decrease in plasmin activity. Hypofibrinolysis and cholesterol accumulation are a direct consequence of the presence of Lp(a) on the surface of fibrin and cell membranes: apo(a) inhibits plasmin formation and the LDL components favor cholesterol accumulation. **FIG 13.**

FIG 13 : ATHEROTHROMBOGENESIS BY LIPOPROTEIN(a)



Growth and proliferation of vascular smooth muscle cells are inhibited by active TGF-ß, a growth factor secreted in latent form and activated by plasmin [66]. It has been recently shown that **Lp(a) inhibits the generation of TGF-ß** [67] and that the generation of plasmin and thereby the activation of TGF-ß are decreased in transgenic mice expressing human apo(a) [68]. Insufficient activation of TGF-ß may result in migration and proliferation of smooth muscle cells into the intima, an important mechanism in atheroma plaque formation. **FIG 14**

FIG 14 : ATHEROMA FORMATION BY LIPOPROTEIN(a)



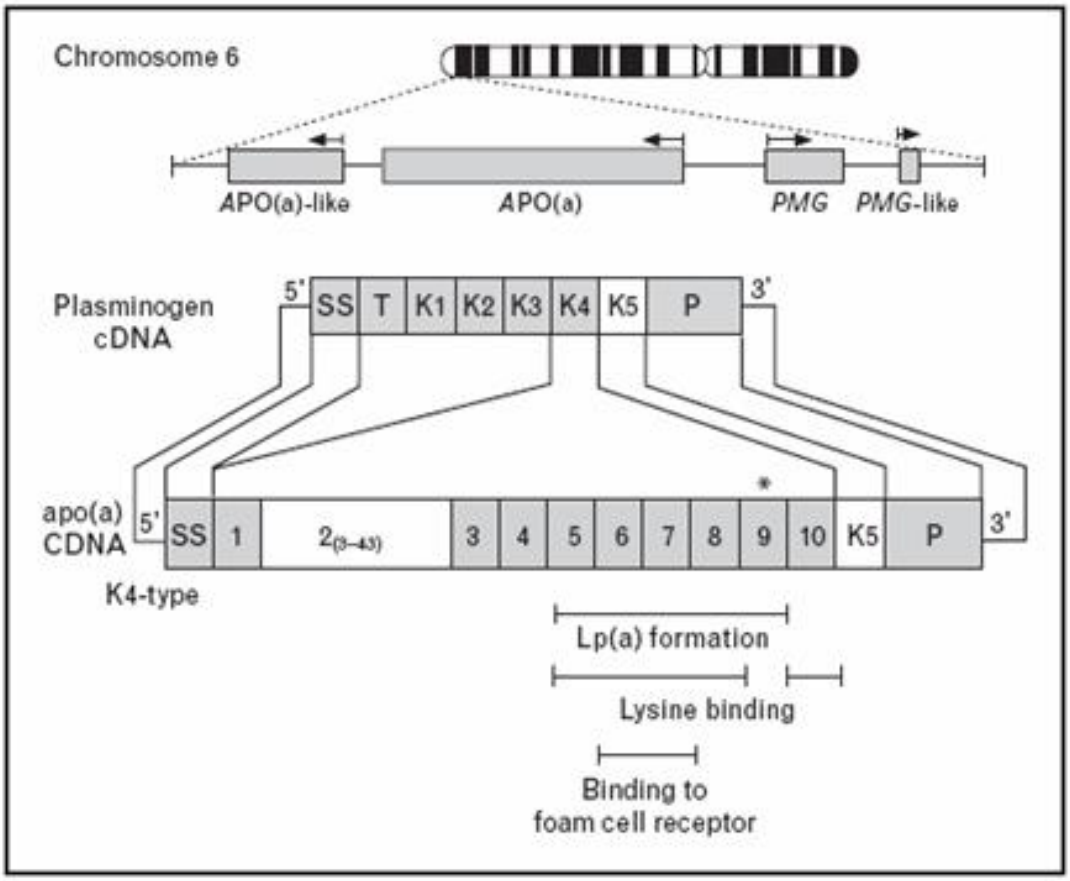
Modification of protein synthesis: Lp(a) may stimulate the expression of PAI-1 and inhibit the synthesis of t-PA by endothelial cells in culture [69]. Thus, inhibition of t-PA by PAI-1 and low t-PA antigen levels may enhance Lp(a)-dependent hypofibrinolysis by decreasing the amount of t-PA available for the activation of plasminogen.

Binding of Lp(a) to extracellular matrix components: Lp(a) and recombinant apo(a) display high affinity for fibronectin and that Lp(a) may form complexes with proteoglycans or glycosaminoglycans of the extracellular matrix [70]. These interactions are not related to the lysine-binding function of kringle 4 and may contribute to the accumulation of Lp(a) in the vascular wall.

Oxidation of Lp(a): The Lp(a) and LDL particles are sensitive to oxidative processes. Phagocytosis of oxidized Lp(a) and LDL particles results in the formation of foam cells [71].

Genetic polymorphism and functional heterogeneity of Lp(a): The circulating concentration of Lp(a) is mainly regulated by the apo(a) gene [72]. **FIG 15.** The size of each allele varies as a function of the number of repetitive sequences encoding kringle 4 type 2. In general, the smaller this hypervariable region and therefore the size of the apo(a) isoform, the higher the plasma concentration of Lp(a).

FIG 15 : GENETIC DETERMINATION OF LIPOPROTEIN(a) FUNCTION



HYPERTENSION AND ATHEROSCLEROSIS

The pathologic influence of hypertension on the development of atherosclerosis is complex, the genetic makeup of the individual, behavioral tendencies (e.g., smoking), and environmental influences all shape the risk for development of atherosclerotic plaque. mouse models of hypertension exhibit increased atherosclerotic lesion size, although there are some exceptions.[73] In addition, there are several reports of reduction in blood pressure resulting in reduced atherosclerosis.

Hypertension and hypercholesterolemia interact strongly in promotion of atherosclerosis. The principal components of blood pressure consist of a steady component (mean arterial pressure) and a pulsatile component (pulse pressure). As large artery stiffness increases in middle aged and elderly subjects, systolic pressure rises and diastolic pressure falls (isolated systolic hypertension) with a resulting increase in pulse pressure[74].

One of the strongest correlations between blood pressure and atherosclerosis is perturbations in the renin-angiotensin system. This hormonal system is responsible for homeostatic control of arterial pressure, tissue perfusion, and extracellular volume. The juxtaglomerular cells located in each nephron produce renin, which converts angiotensinogen (produced mainly in the liver) to angiotensin I. Angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II, which is a potent vasoconstrictor and mediator of aldosterone secretion.

Studies of hyperlipidemic mice in which angiotensin II was chronically infused showed promotion of atherosclerosis independent of changes in arterial blood pressure[75].

Endothelial dysfunction occurs early in the atherosclerotic process, and hypertension is associated with endothelial dysfunction in the coronary, renal, and peripheral

circulations. Studies in animals and humans using an agonist-induced vasodilator response show that this response is blunted in the setting of hypertension.[76]

One key molecule that is partially responsible for the vasodilator response and is involved in maintaining normal endothelial function is nitric oxide (NO). A reduction in endothelial-derived NO may result in not only a reduced vasodilator response but also a proinflammatory, prothrombotic, and procoagulant phenotype[77].

Renal artery stenosis is most commonly due to atherosclerotic disease and may lead to renovascular hypertension. The consequence of the reduction in blood flow to the kidney is activation of the renin-angiotensin-aldosterone system and possibly ischemic nephropathy. One proposed hypothesis is that some of the humoral factors activated by renal artery stenosis may accelerate the atherosclerotic process.[78] Experimental studies of pigs with hypertension secondary to renal artery stenosis showed that increased oxidative stress resulted from this condition and was a stimulus for atherosclerosis independent of cholesterol levels.[79]

HOMOCYSTEINE AND ATHEROSCLEROSIS

It is a metabolic product of amino acid methionine and is found to have a direct effect on the vascular endothelium because it facilitates the formation of oxidized LDL which promotes atherogenesis. It is also said to be thrombotic and to increase collagen production in the extracellular matrix and promotion of smooth muscle cell growth[80].

Prothrombotic effects of homocysteine have been described such as down regulation of thrombomodulin on endothelial cells[81] and upregulation of tissue factor on both endothelial cells and macrophages[82]. Studies have shown relation between

hyperhomocysteinemia and premature disease in coronary, cerebral, and peripheral arteries. It may also enhance the development of atherosclerotic plaques by the following processes:

- Generation of superoxide/ H_2O_2 (damage endothelium)
- Enhance blood coagulation (directly activate platelet aggregation)
- Inhibiting dilation of small arteries (increased vulnerability to clot/ plaque)
- Promote arterial smooth muscle cell proliferation
- It may interact with LDL to form LDL-Homocysteine-thiolactone aggregates, which are taken up by the macrophages, incorporated into foam cells, and become early atherosclerotic plaques.

Daily supplementation of folic acid, vitamin B6, vitamin B12, lower serum homocysteine concentrations and has been reported to decrease plaque area of patients. Women who took maximal levels of these agents had half the risk of having a myocardial infarction compared to those with the lowest intake[82]

HEMOSTATIC FACTORS

Several systemic haemostatic factors including fibrinogen, factor VII, Plasminogen Activator Inhibitor-I, Tissue Plasminogen activator and platelets have been identified as determinants of future coronary heart events[83]. The most powerful and most consistent predictor coronary heart disease among the haemostatic factors- fibrinogen is strongly related to smoking, diabetes and C- reactive protein (an acute phase reactant like fibrinogen) all of which are strong consistent, and independent predictors of coronary heart disease[84]

CYTOKINES AND ATHEROSCLEROSIS

Atherosclerosis is a chronic disease of the arterial wall where both innate and adaptive immunoinflammatory mechanisms are involved. Inflammation is central to all stages of atherosclerosis[85].

Cytokines are defined as a group of protein cell regulators, variously called lymphokines, monokines, interleukins, interferons ("chemokines"), which are produced by a wide variety of cells in the body, play an important role in many physiological responses, are involved in the pathophysiology of a range of diseases, and have therapeutic potential[86].

The cytokines consist of more than 50 secreted factors involved in intercellular communication, which regulate fundamental biological processes including body growth, lactation, adiposity, and hematopoiesis[87]. Cytokines are clustered into several classes: interleukins, tumor necrosis factors (TNF), interferons (IFN), colony stimulating factors (CSF), transforming growth factors (TGF), and chemokines. They are especially important for regulating inflammatory and immune responses and have crucial functions in controlling both innate and adaptive immunity.

The predominant actors in adaptive immunity, helper- T (Th) cells, have been categorized on the basis of the pattern of cytokines that they can secrete, resulting in either a cell-mediated immune response (Th1) associated with IL-2 and IFN-γ secretion, or a humoral immune response (Th2), associated with IL-4, IL-5, IL-10, and IL-13 secretion.

The classification of cytokines is given in the **table-IV**

TABLE IV : CLASSIFICATION OF CYTOKINES

Cytokines	Receptors	Signaling Pathways	
	Class I cytokines		
IL-6/IL-12 family			
Neuropoietic cytokines			
CLC, CNTF, CT-1, LIF	LIFR +gp130	JAK1/JAK2/Tyk2	STAT3
OSM	OSMR +gp130	JAK1/JAK2/Tyk2	STAT3
IL-31	gp130-like receptor (GPL)	JAK1	STAT3/STAT5
Hematopietic cytokines			
G-CSF	gcfsR	JAK1/JAK2/Tyk2	STAT3
IL-6	IL-6Rα +gp130	JAK1/JAK2/Tyk2	STAT3
IL-11	IL-11Rα +gp130	JAK1/JAK2/Tyk2	STAT3
IL-12α	IL-12Rβ2	JAK2/Tyk2	STAT3
IL-23α	IL-23R	JAK2	STAT3
IL-27α	IL-27Rβ	?	?
γ-Chain users (IL-2 family)			
IL-2, IL-15	IL-2Rβ	JAK1/JAK3	STAT5
IL-7, TSLP	IL-7Rα	JAK1/JAK3	STAT5
IL-9	IL-9Rα	JAK1/JAK3	STAT5
IL-21	IL-21Rα	JAK1/JAK3	?
IL-4 family			
IL-4, IL-13	IL-4Rα	JAK1	STAT5
IL-3, GM-CSF, IL-5	IL-3Rβ	JAK2	STAT5
	Class II cytokines		
IFN family			
Type I			
IFN-α, IFN-β	IFNAR1 + IFNAR2	JAK1/Tyk2	STAT1/STAT2
IFN-λ1 (IL-28A), IFN-λ2 (IL-28B), IFN-λ3 (IL-29)	IFNλR1(IL-28Rα) + IL-10R2	JAK1/Tyk2	STAT1/STAT2
Type II			
IFN-γ	IFN-GR1 + IFNGR2	JAK1/JAK2	STAT1
IL-10-related cytokine family			
IL-10	IL-10R1 + IL-10R2	JAK1/Tyk2	STAT1/STAT3/STAT5
IL-19	IL-20R1 + IL-20R2	JAK1/Tyk2	STAT3
IL-20	IL-20R1 + IL-20R2	JAK1/Tyk2	STAT3
IL-22	IL-22R1 + IL-10R2	JAK1/Tyk2	STAT1/STAT3/STAT5
IL-24	IL20R1 + IL-22R1	JAK1/Tyk2	STAT3
IL-26	IL-20R1 + IL-10R2	JAK1/Tyk2	STAT1/STAT3
	TNF superfamily		
TNF-α	TNFR1(p55)/TNFR2(p75)		NF-κB/JNK/p38/ERK
Lymphotoxinα	TNFR1(p55)/TNFR2(p75)		NF-κB/JNK/p38/ERK
FasL	Fas		NF-κB/JNK
CD40L(CD154)	CD40		NF-κB/JNK
RANKL	RANK		NF-κB/JNK/p38/ERK
TRAIL	TRAIL-R		NF-κB/JNK/p38/ERK
	TGF family		
TGF-β1/2/3	TGF-β-R1/ TGF-β-RII		Smad2/Smad3
Activin A	ActRI/ActRII		Smad2/Smad3
	IL-1 family		
IL-1	IL-1RI/IL-1RAcP		NF-κB/JNK/p38/ERK
IL-18	IL-18 Rα/IL-18Rβ		NF-κB/JNK/p38
IL-32	?		NF-κB/p38
IL-33	ST2		NF-κB/p38/ERK

CLC, CT-1-like factor; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; OSM, oncostatin M; G-CSF, granulocyte-colony stimulating factor; TSLP, thymic stromal lymphopoietin; RANK, receptor activator of NF-κB; RANKL, RANK ligand; ActRI, activin type I receptor; IL-1RAcP, IL-1 receptor accessory protein.

Cytokines are categorized according to the structural homology of their receptors as class I or class II cytokines [88] . Most ILs, CSFs, and IFNs belong to one of these two classes of cytokines, which mediate their effects through the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway.

Three other major cytokine families encompass the IL-1 family (including IL-1α, IL-1β, IL-1ra, and IL-18), TNF family, and TGF-β superfamily . IL-1 and TNF family members activate the nuclear factor-κB (NF-κB) and mitogen activated protein (MAP) kinase signaling pathways, while TGF-β superfamily members activate signaling proteins from the smad family.

Table –V shows the list of pro and anti atherogenic cytokines, IL-1 and TGF beta deserve special mention as the chief anti atherogenic cytokines.

TABLE V : PRO ATHEROGENIC AND ANTI ATHEROGENIC CYTOKINES

Proatherogenic Cytokines	Antiatherogenic Cytokines
TNFR family	
TNF-α	
Lymphotoxin	
Osteoprotegerin	
CD40L	
IL-1 family	
IL-1	IL-1ra
IL-18	IL-18BP
Class I cytokines	
IL-2	
IL-4	
IL-6	IL-6
IL-12	IL-9
Class II cytokines	
IFN-γ	IL-10
Hematopoietic factors	
M-CSF	
Chemokines/chemokine receptors	
IL-8/CXCR2	
MCP-1/CCR2	
Fractalkine/CX3CR1	
RANTES	
MIF	
Bone-associated cytokines	
Osteopontin	
TGF-β family	TGF-β

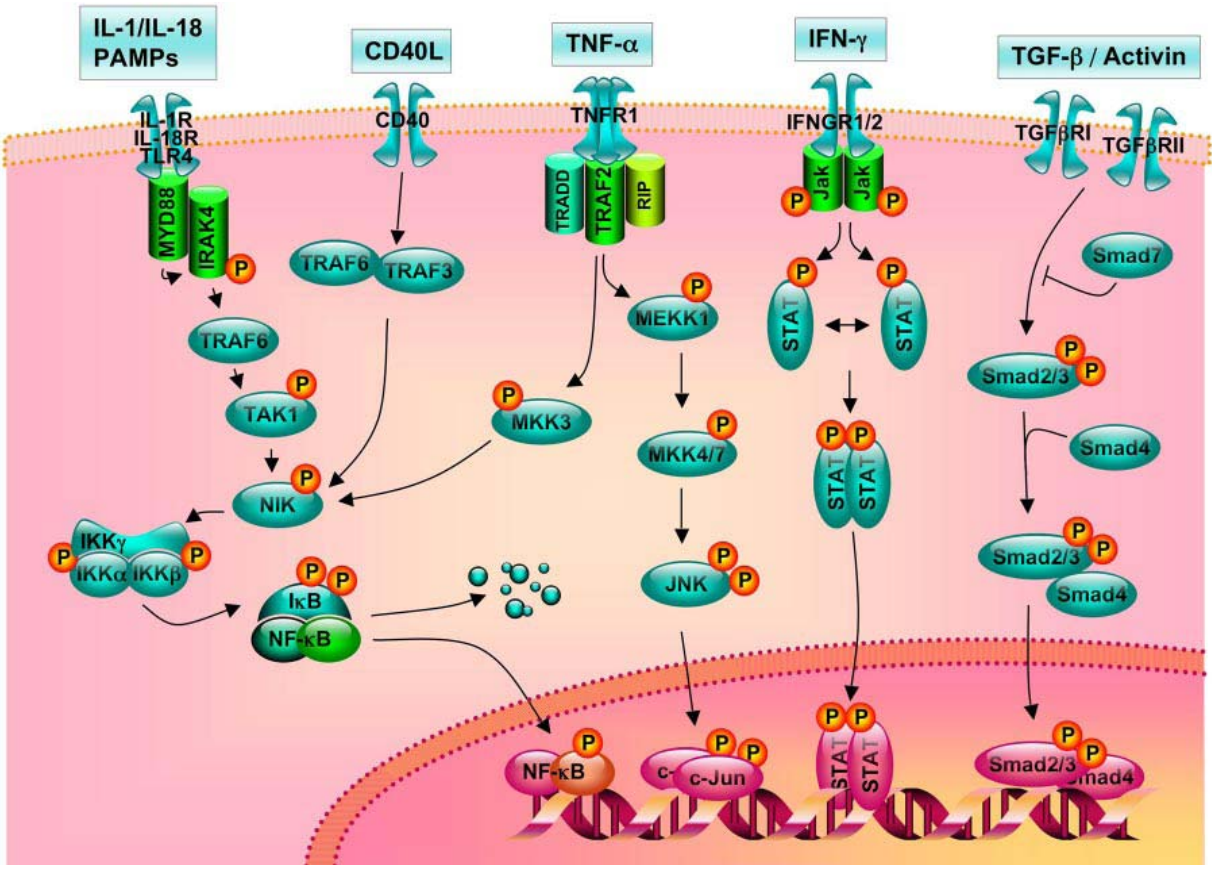
TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor.

Biological Effects of Cytokines

- Effects on endothelial permeability[89]
- Activation of adhesion molecule expression[90]
- Induction of chemokine release[91]
- Modulation of scavenger receptor expression[92]
- Modulation of lipid metabolism[93]
- Activation of 15-lipoxygenase expression in cultured macrophages[94]
- Effect SMC migration/ proliferation[95]
- Regulation of immune response (Th1/Th2/Treg)[96]
- Conversion of CD4+ naïve cells to active CD4+ regulatory cells[97]
- Oxidation of LDL (induction of cell oxidative stress)[98]
- Stimulation of MMP expression[99]
- Modulation of extracellular matrix expression[100]
- Modulation of endothelial SMC progenitor differentiation[101]
- Regulation of neo vessel formation[102]
- Promotion of intra plaque neovascularization[103]
- Induction of apoptosis[104]
- Stimulation of micro particle release[105]
- Modulation of endothelial pro coagulant activity[106]
- Modulation of fibrinolysis (PAI-1)[107]

Fig 16 shows the **principal signaling pathways involved in atherogenesis** .

FIG 16: PRINCIPAL SIGNALING PATHWAYS INVOLVED IN ATHEROGENESIS

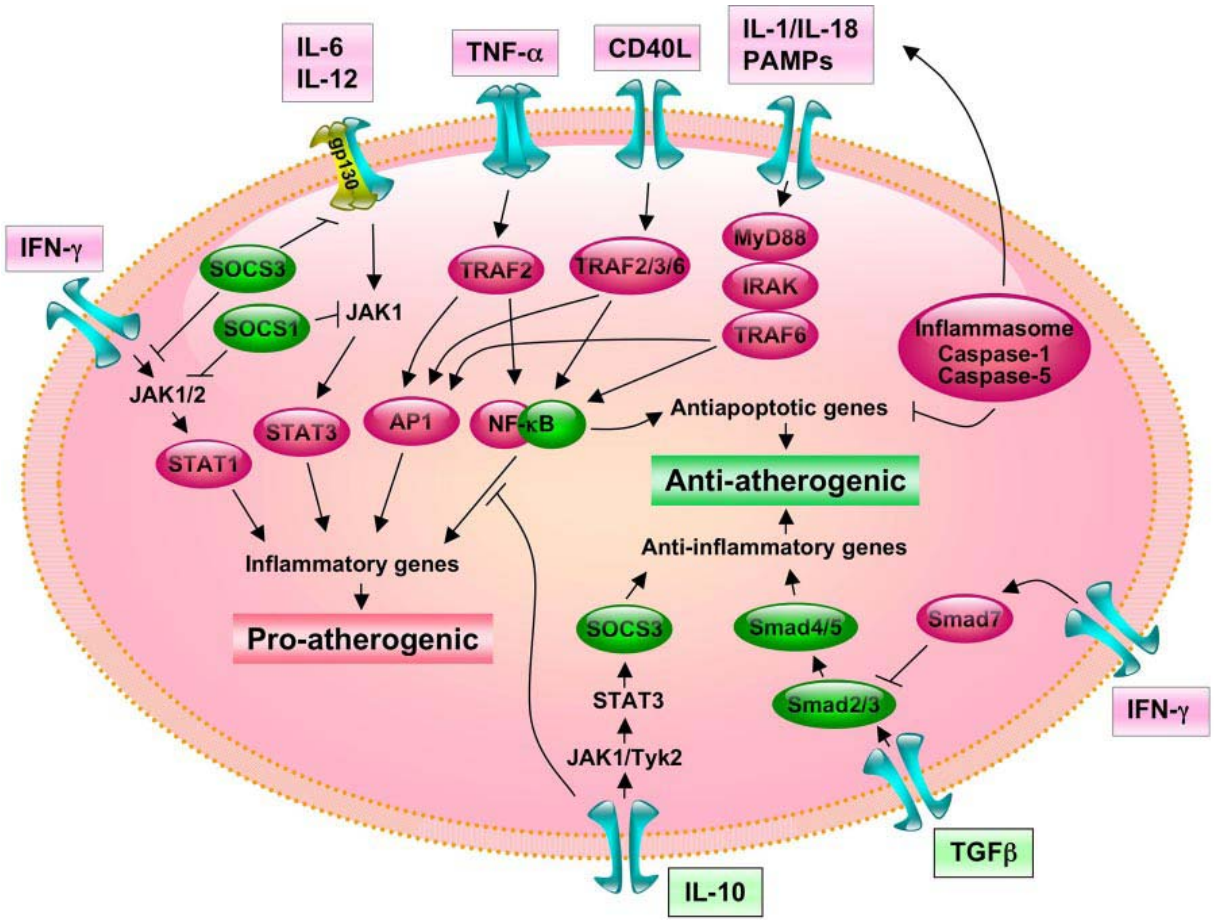


Pro inflammatory cytokines (IL-1, IL-18) and pathogens (known as pathogen associated molecular patterns PAMPs) and non pathogen activators of TLR, act through distinct signaling pathways that converge on the activation of NF-κB. MyD88 functions as an adapter molecule between the receptors of TLR or IL-1 R and downstream signaling kinases. Following association of MyD88 with IL-1-associated kinase IRAK-4, IRAK-4 is autophosphorylated, dissociates from the receptor complex, and interacts with TNF-receptor-associated factor-6 (TRAF-6), which also mediates CD40 signaling. Once activated, TRAF6 associates with the MAP3 kinase TAK1 [108]. From TAK1, two signaling pathways diverge; one ultimately leads to NF-κB activation and the other to MAP kinase activation. In its inactive form, NF-κB is bound to inhibitor of κB (IK-κBα/β) in the cytoplasm and consists of an IκB kinase (IKK) complex containing two kinases IKKα and IKKβ, and the regulatory protein IKKγ (also named NEMO); IKK activation initiates IκBα/β phosphorylation. Phosphorylated IκB is then ubiquitinated, leading to its degradation by the 26S proteasome. This releases NF-κB dimers from the cytoplasmic NF-κB-IκB complex, allowing them to translocate to the nucleus. JNK phosphorylation is mediated by two MAPK kinases (MAPKKs), MKK4 and MKK7, that they can cooperatively activate JNK. Both kinases are required for full activation of JNK by environmental stressors, and MKK7 is essential for JNK activation by TNR engagement. Tyrosine phosphorylation activates the cytosolic inactive STATs, resulting in their nuclear translocation and gene activation. This pathway was originally found to be activated by IFNs, but a number of cytokines, growth factors, and hormonal factors also activate JAK and/or STAT proteins. IFN-γ utilizes JAK1 and JAK2, and usually activates STAT1. TGF-β-triggered signals are transduced by proteins belonging to the

Smad (for vertebrate homologs of *Sma* and *Mad*) family. The type I receptor recognizes and phosphorylates Smad2 and Smad3, which associates with Smad4, forming complexes that participate in DNA binding and recruitment of transcription factors. Smad3 may also have antagonistic properties, as it plays a critical role in TGF-β-dependent repression of vascular inflammation by inhibiting AP-1 activity. Smad7 inhibits Smad2 and Smad3 phosphorylation.

Fig17 shows **cross talks between pro atherogenic and anti atherogenic signal transduction pathways**

FIG 17 : Cross talks between pro atherogenic and anti atherogenic signal transduction pathways



Inhibitory Smads such as Smad7 downstream of IFN-γ signaling associate with activated receptors and interfere with Smad2 and Smad3 binding. It is noteworthy that like IFN-γ, the anti-inflammatory cytokine IL-10 also activates JAK and/or STAT proteins. However, the IL-10/IL-10R interaction activates JAK1 and Tyk2, leading to STAT3 and SOCS3 activation, which is central for the anti-inflammatory responses of IL-10 in macrophages. The inflammasome may be a central link between apoptosis and inflammation in pathological conditions. NF-κB may have a dual role in atherosclerosis, being proatherogenic through its proinflammatory properties, and antiatherogenic through its antiapoptotic activities.

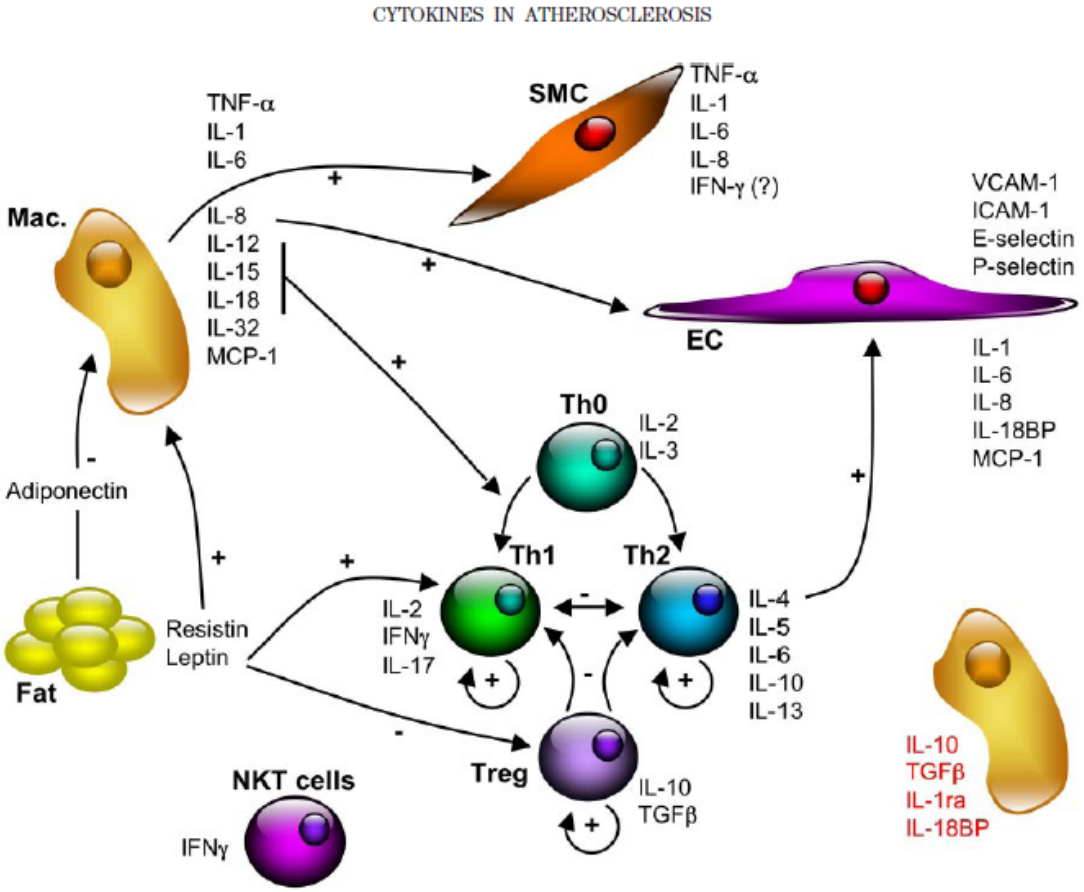
Table VI shows **primary and secondary triggers for the release of cytokines in atherosclerosis**

TABLE VI : PRIMARY AND SECONDARY TRIGGERS FOR CYTOKINE RELEASE IN ATHEROSCLEROSIS

Primary: bioactive lipid mediators
Oxidized low-density lipoprotein
4-Hydroxy-2-nonenal (4HNE)
Oxysterols
Oxidized phospholipids (oxPL)
Lysophosphatidylcholine (lysoPC)
Oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (oxPAPC)
Platelet activating factor (PAF)
Secondary
Heat shock proteins
Immune complexes
Infectious agents
Defective clearance of apoptotic cells
Matrix metalloproteinases
Inflammasome
Oxygen radicals
Angiotensin II
Advanced glycated end products
Proinflammatory cytokines
Toll-like receptor endogenous ligands
Mechanical factors
Hypertension
Disturbed flow
Adipokines
Leptin
Resistin
Platelet products

Fig18 shows **the cytokines involved in atherosclerosis**

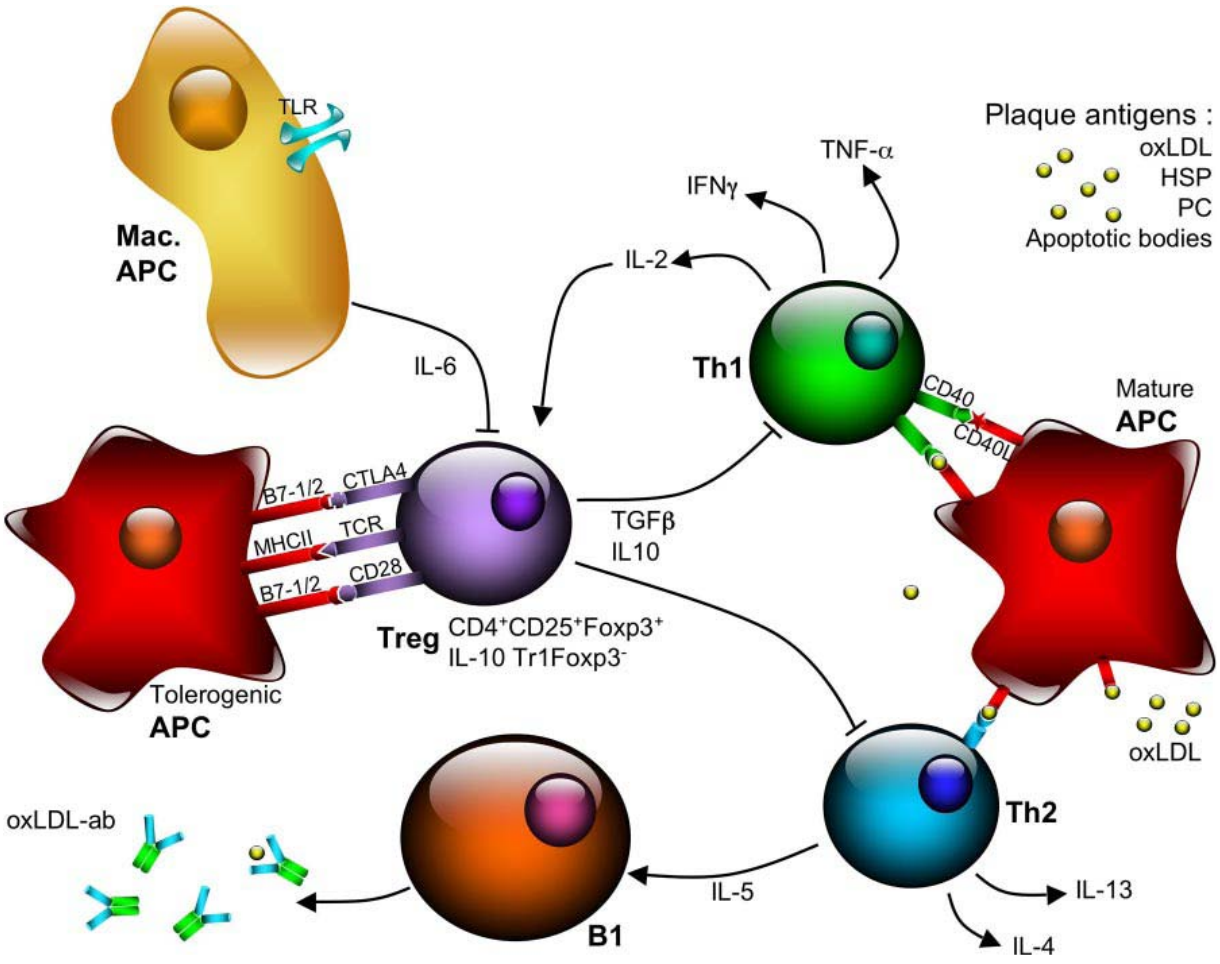
FIG 18 : CYTOKINES INVOLVED IN ATHEROSCLEROSIS



Cytokines are produced by several cell types, including inflammatory and vascular cells, as well as adipocytes. IL-12 and IL-18 produced by macrophages are potent inducers of IFN-γ and promote the differentiation of naive T cells into proatherogenic Th1 cells. Macrophage or macrophage-derived cytokines also activate smooth muscle cells (SMC) and endothelial cells (EC) to produce an array of proinflammatory mediators. On the other hand, the anti-inflammatory cytokines IL-10 and TGF-β, also produced by macrophages, promote antiatherogenic Treg cell differentiation. Other anti-inflammatory mediators with potent antiatherogenic properties include IL-1 receptor antagonist (IL-1ra) and IL-18 binding protein (IL-18BP). Interestingly, IL-4, the prototype of Th2 cells, has proinflammatory properties on EC. Adipocytes produce both pro- and anti-inflammatory mediators. Leptin activates Th1 cells but inhibits Treg cell function. Adiponectin has been shown to dampen macrophage activation.

Fig 19 shows **Development and maintenance of pathogenic and regulatory immunity in atherosclerosis**

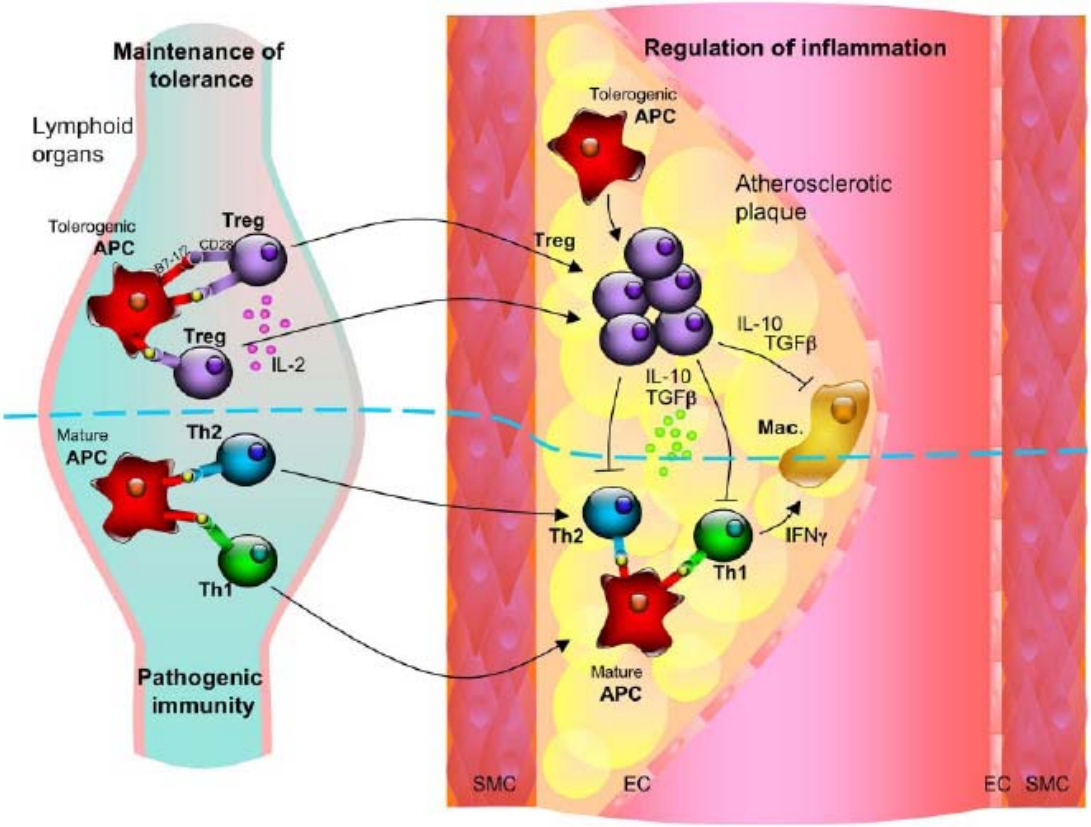
FIG 19 : Development and maintenance of pathogenic and regulatory Immunity in atherosclerosis



Candidate antigens [oxidized lipoproteins (oxLDL), heat shock proteins (HSP), phosphorylcholine (PC), apoptotic bodies may induce Th1, Th2, or both Th1 and Th2 pathogenic responses. Maturation of the antigen presenting cell (APC) is necessary for T-cell priming. The CD40/CD40L pathway is critical for Th1 differentiation. IL-6 and IL-13 contribute to the induction of Th2 cell type. Both IFN-γ (Th1) and IL-4 (Th2) have been shown to promote atherogenesis. Production of IL-5 by Th2 cells is important for protective antibody production by B lymphocytes in response to immunization with oxLDL. Distinct subsets of APCs, called "tolerogenic" cells, induce the differentiation of the regulatory T-cell subset (Treg). The development of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells occurs in the thymus and requires TCR and CD28 engagement among other yet unknown factors. Interestingly, production of IL-2 by pathogenic Th1 cells is essential for the survival and maintenance of this Treg subset in the periphery. Other Treg cells of the Tr1 type may not express Foxp3 and are mainly induced in the periphery in response to antigen stimulation. Both types of Treg cells use IL-10 and/or TGF-β to suppress the proliferation of pathogenic T cells in vivo. IL-6, produced in response to TLR stimulation, may contribute to the inhibition of Treg function.

FIG. 20 shows **Local and systemic effects of pathogenic and regulatory cells in atherosclerosis.**

FIG 20 : Local and systemic effects of pathogenic and regulatory cells in atherosclerosis.



Lymphoid organs are specialized in antigen presentation and may be the major site of pathogenic or tolerogenic antigen presentation and T-cell priming in atherosclerosis. Antigen presentation may also occur within the atherosclerotic plaque, which is rich in cells with antigen-presenting capacity (macrophages and dendritic cells). Continuous trafficking of immune cells between the inflammed atherosclerotic artery and the lymphoid organs may be necessary to mount an adaptive immune response. CD28 engagement and IL-2 production by pathogenic T cells are required for Treg cell survival and maintenance in the periphery. Treg cells suppress the pathogenic response through IL-10, TGF-β, and/or cell-cell contact-dependent mechanisms. The precise mechanisms that drive a pathogenic or a regulatory immune response in atherosclerosis are currently unknown.

TRANSFORMING GROWTH FACTOR- β:

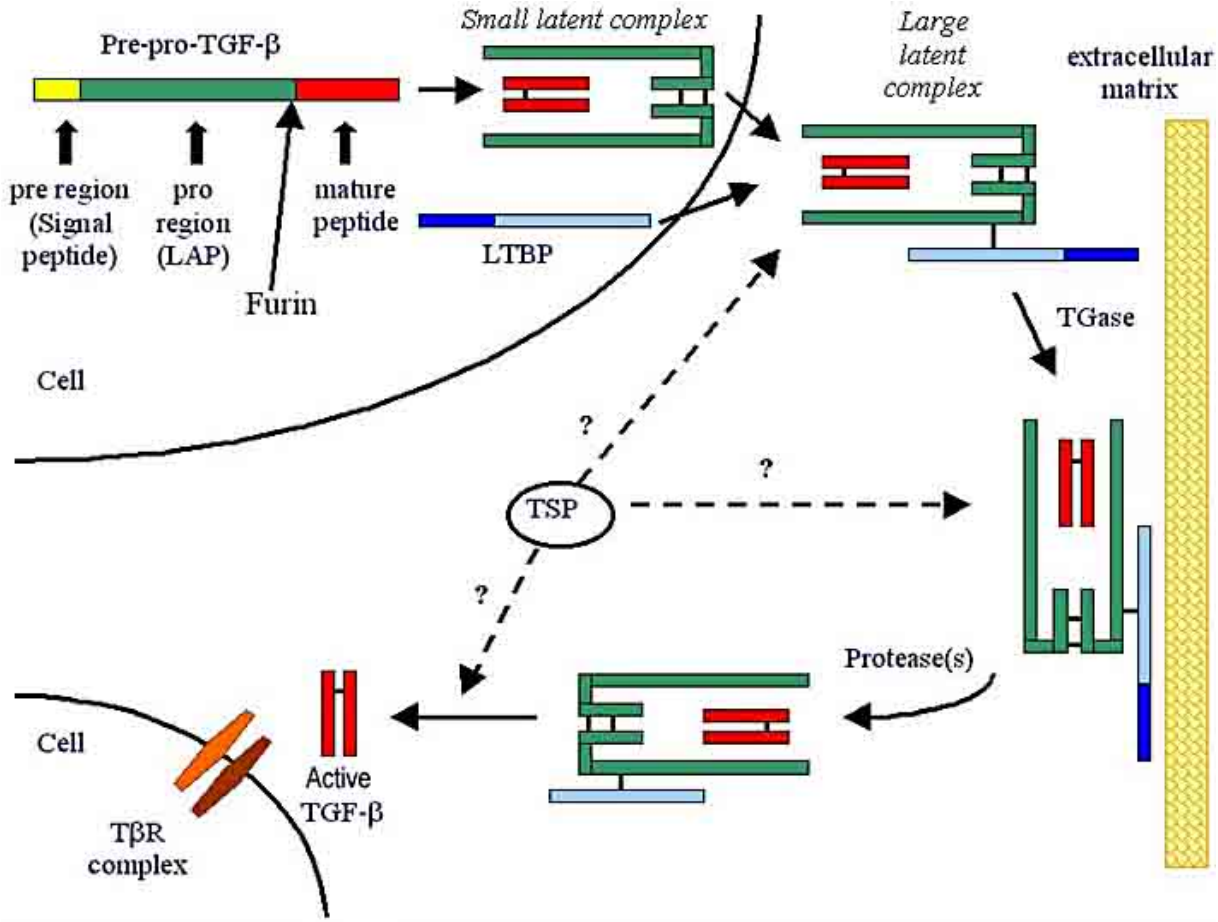
TGF-β is a secreted protein that exists in at least three isoforms called TGF-β1, TGF-β2 and TGF-β3. the transforming growth factor beta superfamily, includes inhibins, activin, anti-müllerian hormone, bone morphogenetic protein.

Structure: TGF-β1 contains 390 amino acids and TGF-β2 and TGF-β3 each contain 412 amino acids. They each have an N-terminal signal peptide of 20-30 amino acids that they require for secretion from a cell, a pro-region (called latency associated peptide or LAP), and a 112-114 amino acid c- terminal region that becomes the *mature* TGF-β molecule following its release from the pro-region by proteolytic cleavage. The mature TGF-β protein dimerizes to produce a 25 KDa active molecule with many conserved structural motifs. TGF-β has nine cysteine residues that are conserved among its family; eight form disulphide bonds within the molecule to create a cysteine knot structure characteristic of

the TGF-β superfamily while the ninth cysteine forms a bond with the ninth cysteine of another TGF-β molecule to produce the dimer**.**

FIG 21. shows the **synthesis of mature TGF beta**.

FIG 21 : SYNTHESIS OF MATURE TGF β



LAP- Latency Associated Peptide

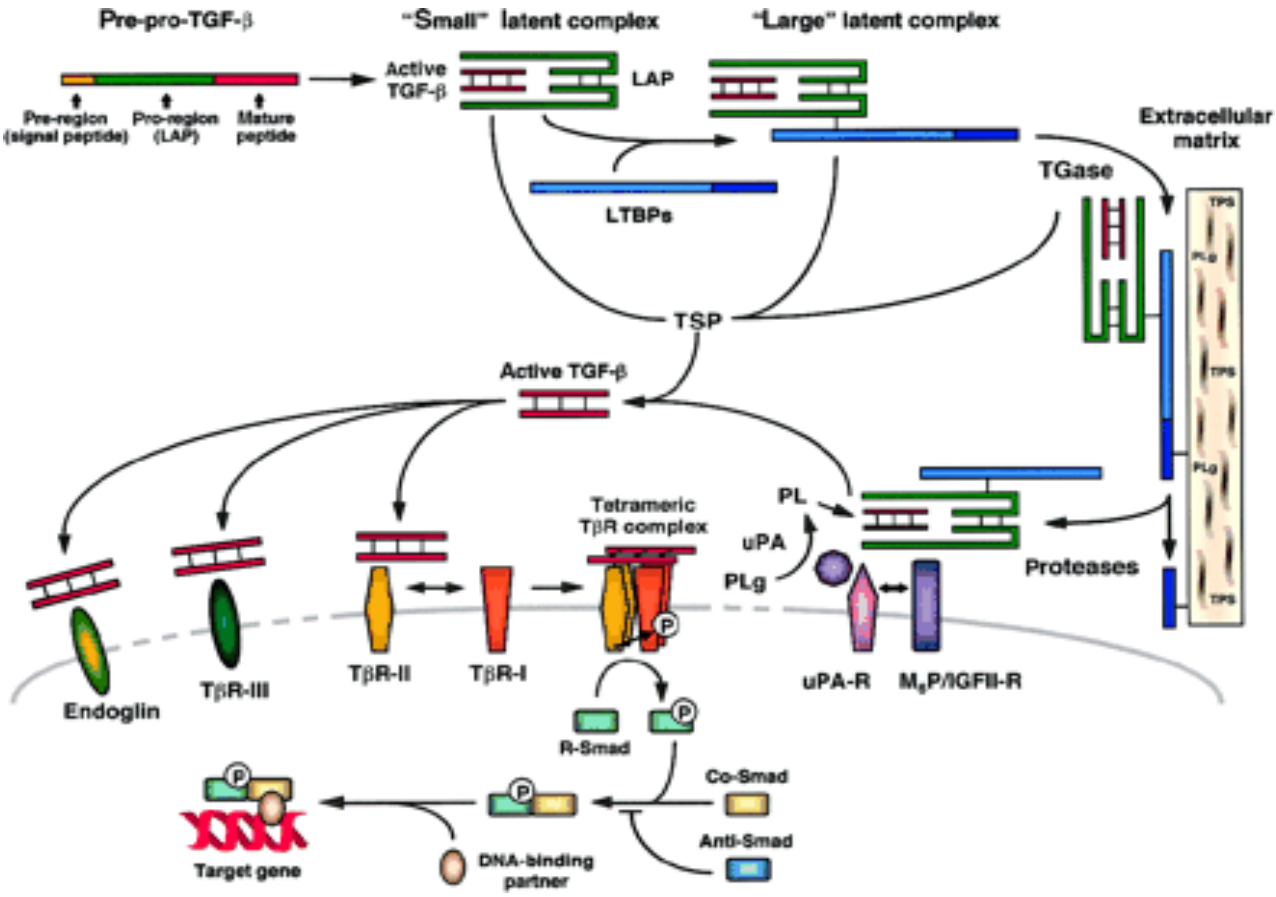
LTBP- Latent TGF beta Binding Protein

TGF-β is a potent anti-inflammatory, immunosuppressive and pro-fibrotic cytokine, with major effects on the biology of SMC [109]. TGF-β1- deficient mice die in utero or in the perinatal period because of widespread uncontrolled inflammation [110]. The anti-inflammatory and profibrotic properties of TGF-β are highly suggestive of a potential antiatherogenic role for this cytokine. Indeed, serum active TGF-β is markedly depressed in patients with advanced atherosclerosis [111], and that TGF-β1 heterozygous mice fed a cholate-supplemented atherogenic diet displayed increased endothelial activation and macrophage infiltration in the aortic sinus [112]. The critical role of TGF-β for SMC matrix production and Plaque stability in atherosclerosis was thereafter demonstrated by studies using apoE_/_ mice. Treatment of apoE_/_ mice with neutralizing antibodies to TGF-β1, TGF-β2, and TGF-β3 accelerates the development of atherosclerosis, with lesions displaying increased inflammatory cells and decreased collagen content [113]. TGF-β may therefore reduce inflammation but also contributes to matrix production within lesions. In agreement with this hypothesis, treatment of apoE_/_ mice with a soluble TGF-β-receptor II protein (TGF-RII:Fc) that inhibits TGF-β signaling resulted in larger plaques, with an increased frequency of macrophages and T cells and decreased collagen content in the atherosclerotic lesions [114]. Moreover, intraplaque hemorrhages were frequently observed. specific inhibition of TGF-β signaling in T cells leads to the development of atherosclerotic Plaques with a phenotype that may potentially increase plaque vulnerability to rupture, strongly suggesting an important protective role of endogenous T-cell TGF-β activity against vulnerability to atherosclerosis[115]. Bone marrow transplantation from transgenic mice that express a dominant negative TGF-β receptor type II under a T-cell-specific promoter into LDLr_/_ mice resulted in increased

differentiation of spleen-derived T cells toward both Th1 and Th2 phenotypes [116]. Moreover, atherosclerotic plaques of these mice showed increased T-cell infiltration and expression of MHC class II, along with a decrease in SMC and collagen content. Consistent with these findings, apoE_/_ mice with disrupted TGF-β signaling in T cells exhibited markedly larger atherosclerotic lesions, with a _100-fold increase in aortic IFN-γ expression compared with apoE_/_ Littermates [115]. The important role of T-cell-TGF-β signaling in atherosclerosis suggests that regulatory pathways in adaptive immunity are essential in modulation of the development and progression of the disease.

TGF beta functions are exerted through the smad pathway. **Fig 22.** TGF-β dimers bind to a type II receptor which recruits and phosphorylates a type I receptor. The type I receptor then recruits and phosphorylates a receptor regulated SMAD (R-SMAD). SMAD3, an R-SMAD, has been implicated in inducing apoptosis. The R-SMAD then binds to the common SMAD (coSMAD) SMAD4 and forms a heterodimeric complex. This complex then enters the cell nucleus where it acts as a transcription factor for various genes, including those to activate the mitogen-activated protein kinase 8 pathway, which triggers apoptosis.

Fig 22: TGF β – Structure, Latency, Activation and Receptor



LAP - latency-associated peptide
LTBP- latent TGF-beta binding protein
M6P/IGFII-R- mannose-6-phosphate/type II insulin-like growth factor receptor
PLg- plasminogen
PL- plasmin
Smad TGF-beta signal transduction proteins
Anti-Smad- antagonistic Smad
Co-Smad- common-partner Smad
R-Smad- receptor-regulated Smad
TGase- transglutaminase
TbetaR-I, -II, -III- TGF-beta receptor type I, II, III
TSP- thrombospondin
uPA- urokinase plasminogen activator
uPA-R- uPA receptor.

Salient Functions :

- TGF beta plays a crucial role in the regulation of cell cycle at G1 phase
- Regulates the immune system through the $Foxp3^{+}$ regulatory T cells.
- Inhibits the activation of lymphocytes and monocyte derived phagocytes
- Vital role in cancer, heart disease, renal disease, hypertension, inflammation, connective tissue diseases like Marfan syndrome, etc.

AIM OF THE STUDY

AIM OF THE STUDY

Atherosclerosis is a multifactorial disease, the cornerstone being dyslipidemia and oxidative stress. Cardiovascular manifestations of atherosclerosis has been extensively studied, and treatment of dyslipidemia is a major focus in the management of Coronary Artery disease. Inspite of the wide array of drugs prescribed to patients, the response to drugs differ between individuals, so is the lipid profile. This has led to focus upon the genetic predisposition of people to atherosclerosis and the different patterns observed. These non modifiable risk factors have to be analyzed in population belonging to various ethnic origins and the treatment approach should be streamlined according to the population. The risk factors for atherosclerosis among developing countries also differ from those of developed countries.

Peripheral vascular disease has been increasingly gaining importance as a major cause of debilitation and death. While there are voluminous records of studies done on coronary artery and cerebrovascular diseases, Indian studies on peripheral vascular disease appear to be relatively scarce, the dearth more apparent among the south Indian population.

The sinister role of Low density lipoprotein and recently that of lipoprotein(a) , and the protective role of HDL have been well established in Coronary and cerebro vascular disease. The same can be true for Peripheral vascular disease, only that it remains to be ascertained by more number of studies in native Indian population. The pathogenic role of lipoprotein(a) has long been a mystery, more mechanisms of action being discovered as researchers probe into it. Still, Lp(a) has not been approved widely as a conventional

parameter in the lipid profile analysis, and it is not amenable to routine treatment with lipid lowering drugs.

An equally interesting factor is the Transforming Growth Factor beta (TGF-β), which plays an extremely vital role in many of the normal physiologic and also pathologic events of the system. Its association with peripheral vascular disease has not been proved decisively yet. Powerful molecule as it is, the presence or absence of it could be central to the evolution of the atherosclerotic plaque. Its level has been influenced by a wide variety of conditions and factors, one among them is lipoprotein(a).

An insight into these intriguing factors has led to the performance of this study, whose Aims are:

To study the levels of lipoprotein(a) and transforming growth factor-β in atherosclerotic peripheral vascular disease patients from the south Indian population, and to assess the predictive power of Lp(a) as a marker of peripheral vascular disease.

To study the relationship between the levels of Lp(a) and TGF-β and establish the correlation.

To determine the relation of other conventional risk factors of atherosclerosis with Lp(a) in peripheral vascular disease.

To evaluate the possibility of setting a cut off value for Lp(a) and TGF-β, above which PVD risk can be assessed.

MATERIALS AND METHODS

MATERIALS AND METHODS

After obtaining approval from the Institutional Ethical Committee of Madras Medical College and Rajiv Gandhi Govt Gen Hospital, the study was conducted in the Department of Vascular surgery in RGGGH.

STUDY POPULATION

CASES

The study sample comprised 50 unrelated south Indian Peripheral Vascular Disease patients (47 male , 3 female) of Mean age 51.96 +/- 10.1 years. Inclusion criteria was ABPI (Ankle Brachial Pressure Index) less than 0.9 in the affected limb. All patients with systemic hypertension, acute infection and inflammatory disorders, liver or renal disease, thrombotic tendencies and metastatic conditions were excluded. Peripheral vascular disease other than that due to Atherosclerosis was also excluded (eg. Thromboangitis obliterans, autoimmune diseases)

CONTROL SUBJECTS

Controls were recruited from Master health Check-up. Apparently healthy individuals matched for age and sex were included. All of them were free from symptoms and signs of peripheral arterial disease.

Sample collection and Processing

3 mL of blood was collected into a plain tube by Venipuncture after overnight fasting. The blood was allowed to clot and Serum was separated by centrifugation. 0.5

mL of serum was stored in two eppendorf tubes at -20^{o} C for analysis of Lipoprotein (a) and TGF β. The levels of total cholesterol, triglycerides and high density lipoprotein were measured in XL 300 fully automated analyzer by colorimetric methods using commercially available kits within 6 hrs of blood collection.

LIPID PROFILE

The biochemical parameters undertaken for the study were determined using the following methodologies:

ESTIMATION OF TOTAL CHOLESTEROL

Kit used

Erba Mannheim XL system pack

Methodology

CHOD-PAP

Principle

$$\text{Cholesterol esters} \xrightarrow{\text{CE}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{CO}} \text{Cholest- 4-en-3-one + } H_2O_2$$

$$2\ H_2O_2 + \text{Phenol+ 4 AAP} \xrightarrow{\text{POD}} \text{Quinoeimine Dye + 4 } H_2O$$

CE = Cholesterol Esterase

CO = Cholesterol Oxidase

4AAP = 4-amino antipyrine

POD = Peroxidase

The concentration of cholesterol in the sample is directly proportional to the intensity of the red complex (red quinone), which is measured at 505nm.

REAGENT COMPOSITION

Active Ingredients	**Concentration**
Pipes buffer (pH 7.0)	35 mmol/L
Sodium Cholate	0.5 mmol/L
Cholesterol Oxidase	>0.1 U/mL
Cholesterol Esterase	>0.2 U/mL
Peroxidase	>0.8 U/mL
4-amino antipyrine	0.5 mmol/L
Phenol	28 mmol/L

Reagents supplied were ready to use

Calibrator

Total Cholesterol – 200 mg/dL

Assay paramaters

Assay type - 1-Point

Wavelength primary - 505 nm

Wavelength secondary - 0

Sample volume - 3 µL

Reagent volume - 300 µL

Reaction direction - Increasing

Calibration curve - Straight

Reference values

Male : 120 – 250 mg/dL

Female : 110 – 230 mg/dL

ESTIMATION OF TRIGLYCERIDE

Kit Used

Erba Mannheim system pack

Methodology

GPO-ADPS

Principle

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + 4\ \text{AAP} + \text{ADPS} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + H_2O$$

LPL - Lipoprotein Lipase

GK - Glycerol Kinase

ATP - Adenosine Triphosphate

ADP - Adenosine Diphosphate

GPO - Glycerol Phosphate Oxidase

DAP - Dihydroxy Acetone phosphate

ADPS - 4 Chlorophenol

The intensity of red color produced is proportional to the concentration of triglycerides and is measured at 546 nm.

REAGENT COMPOSITION

Active Ingredients	**Concentration**
R1	
Pipes buffer (pH 7.0)	40 mmol/L
4-Amino antipyrine	0.4 mmol/L
ATP	1.5 mmol/L
Mg^{2+}	1.6 mmol/L
Glycerol Kinase	> 66.67 µkat/L
Glycerol 3 phosphate oxidase	> 60.00 µkat/L
R2	
ADPS	0.6 mmol/L
Peroxidase	> 20.00 µkat/L
Lipoprotein Lipase	> 16.67 µkat/L

REAGENT PREPARATION

Reagents supplied were ready for use

CALIBRATOR

Serum Triglyceride – 200 mg/dL

ASSAY PARAMETERS

Assay Type	1 point
Wavelength primary	546 nm
Wavelength secondary	0
Sample volume	3 µL
R1 vol	240 µL
R2 vol	60 µL
Reaction direction	Increasing
Calibration curve	Straight

REFERENCE VALUES

Male : 40 – 160 mg/dL

Female: 35 – 135 mg/dL

ESTIMATION OF HDL CHOLESTEROL

Kit used

Erba Mannheim system pack

Methodology

Immunoinhibition

Principle

Chylomicrons, VLDL, and LDL fractions in plasma are separated from HDL by Immunoinhibition. LDL, VLDL, and chylomicron (CM) react with PVS (Polyvinyl sulfonic acid) and PEGME (Polyethylene glycol methyl ester) and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER). The enzymes selectively react with HDL to produce H_2O_2 which is detected through a Trinder reaction.

REAGENT COMPOSITION

R1

MES buffer	6.5 mmol/L
N,N Bis(4-sulfobutyl)-3-methylaniline	3 mmol/L
Polyvinyl sulfonic acid	50 mg/L
Polyethylene glycol methyl ester	30mL/L
$MgCl_2$	2 mmol/L

EDTA

Detergent

R2

MES buffer (pH 6.5)	50 mmol/L
Cholesterol esterase	5 kU/L
Cholesterol Oxidase	20 kU/L
Peroxidase	kU/L
4-amino antipyrine	0.9 g/L
Detergent	0.5%

REAGENT PREPARATION

Reagents supplied were ready for use

Calibrator

HDL Cholesterol – 60 mg/dL

ASSAY PARAMETERS

Assay type	2 point

Wavelength primary	600 nm
Wavelength secondary	700 nm
Sample volume	3 µL
R1 volume	210 µL
R2 volume	70 µL
Reaction Direction	Increasing
Calibration Curve	Straight

Reference Values

Male : 35.3 to 79.5 mg/dL

Female : 42 to 88 mg/dL

ESTIMATION OF VLDL and LDL CHOLESTEROL

These parameters were calculated using Friedwald's formula given below:

LDL-C = TC-(HDL-C+ VLDL-C)

VLDL-C = TGL/5

ESTIMATION OF LIPOPROTEIN (a)

Kit Used

Lp(a) Turbilatex kit from Spinreact

Methodology

Immunoturbidimetry

Principle

Latex particles coated with antibodies anti-Lp(a) are agglutinated when mixed with samples containing Lp(a). The agglutination causes an absorbance change, dependent upon the Lp(a) contents of sample that can be quantified at 570 nm by comparison from a calibrator of known Lp(a) concentration. The assay was done in a spectrophotometer Helios

Reagents

Diluent (R1) - Glycine buffer 50 mmol/L pH 9.0, Sodium azide 0.95 g/L

Latex (R2) - latex particles coated with mouse monoclonal anti Lp(a), pH 8.2, Sodium Azide 0.95 g/L

Calibrator

Was reconstituted with 1 mL of NaCl (normal saline). Concentration of Lp(a) 100.8 mg/dL. The following dilutions were prepared.

Calibrator Dilution	1	2	3	4	5
Lp(a) calibrator µL NaCl 0.9 g/L µL	0 100	25 75	50 50	75 25	100 0
Factor	0	0.25	0.5	0.75	1.0

Procedure

Assay conditions:

Temperature – 37^{o} C

Wavelength - 570 nm (540-600 nm)

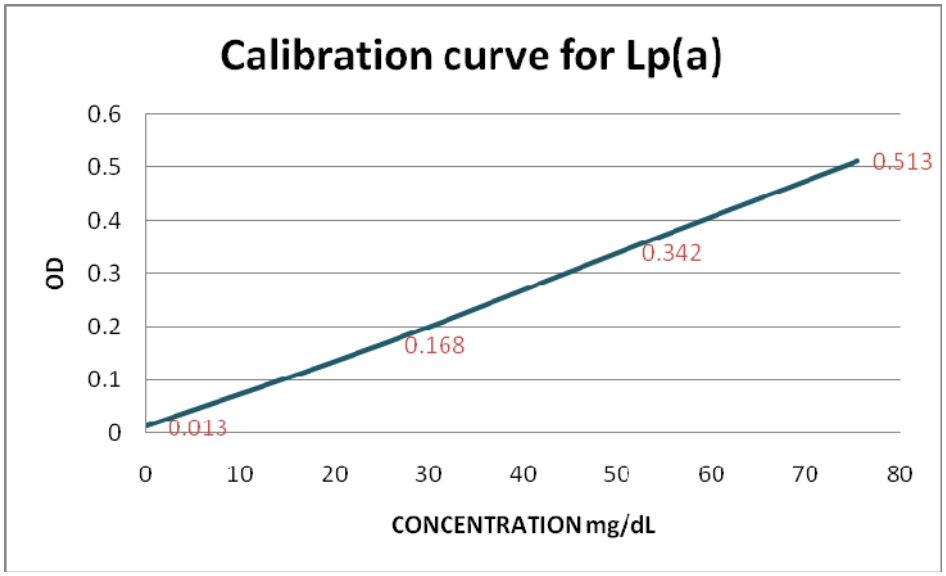
Cuvette light path – 1 cm

R1 volume - 800 µL

R2 volume - 200 µL

Sample/ Calibrator volume – 15 µL

The reagents and sample were mixed well and the absorbance was read immediately (A1) and after 4 minutes (A2).



CALCULATIONS

Calibration curve : The absorbance differences (A_2-A_1) of each Lp(a) calibrator was calculated and the values obtained were plotted against the Lp(a) concentration in a calibration curve. Lp(a) concentration in the sample was calculated by interpolation of its (A_2-A_1) in the calibration curve.

Reference values

Normal values upto 30 mg/dL

ESTIMATION OF TGF β

Kit Used

DRG TGF-β1 ELISA kit

Methodology

Enzyme Immunoassay

Principle

This is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle.

Prior to testing the standards and patient samples are diluted in assay buffer, acidified with HCl and then neutralized with NaOH. Afterwards, the neutralized standards and samples are added to the antibody coated (polyclonal) microtiter wells. After the first incubation the unbound sample material is removed by washing. Then a monoclonal mouse anti TGF-β1 antibody, a biotinilated anti mouse IgG antibody and the Streptavidin-HRP Enzyme complex are incubated in succession. An immunoenzyme sandwich complex is formed.

After incubation the unbound conjugate is washed off. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of TGF β1 in the patient sample.

Reagents

1. **Microtiterwells**, 12x8 (break apart) strips, 96 wells;

Wells coated with anti-TGF-β1 antibody (polyclonal).

2. **Standard (Stock Standard),** 1 vial, 2 mL,

Concentrations: 600 pg/mL

3. **Assay Buffer**, **10X concentrate,** 1 vial, 10 mL,

Concentrations: 0 pg/mL

4. **Antiserum**, 1 vial, 11 mL, ready to use,

monoclonal Mouse anti-TGF-β1

5. **Enzyme Conjugate**, 1 vial, 11 mL, ready to use,

anti Mouse IgG conjugated to Biotin.

6. **Enzyme Complex**, 1 vial, 11 mL, ready to use

Streptavidin Peroxidase

7. **Substrate Solution**, 1 vial, 14 mL, ready to use,

Tetramethylbenzidine (TMB).

8. **Stop Solution**, 1 vial, 14 mL, ready to use,

contains 0.5M H2SO4,

9. **Wash Solution**, 1 vial, 30 mL (40X concentrated),

10. 1 N **HCl,** 1 vial, 3 mL, ready to use, for acidification of the samples.

11. 1 N **NaOH**, 1 vial, 3 mL, ready to use, for neutralization.

The stock standard was kept frozen at -20^{o}C until analysis.

Reagent preparation

All the reagents were brought to room temperature prior to use

Assay Buffer

10 mL of concentrated assay buffer was diluted with 90 mL of deionized water to a final volume of 100 mL working assay buffer.

Standards

Serial dilution of stock standard (600 pg/mL) was prepared as follows:

Description Concentration

Standard A 600 pg/mL

Standard B 1 mL Standard A + 1 mL Assay Buffer **300 pg/mL**

Standard C 1 mL Standard B + 1 mL Assay Buffer **150 pg/mL**

Standard D 1 mL Standard C + 1 mL Assay Buffer **75 pg/mL**

Standard E 1 mL Standard D + 1 mL Assay Buffer **38 pg/mL**

Standard F 1 mL Standard E + 1 mL Assay Buffer **19 pg/mL**

Standard G 2 mL Assay Buffer **0 pg/mL**

Wash solution

30 mL of concentrated wash solution was diluted with 1170 mL of deionized water to a final volume of 1200 mL.

Specimen dilution

Serum and Plasma Samples were diluted **1:50** with *Assay Buffer* prior to testing as follows

10 µL Serum + 490 µL *Assay Buffer* (mixed thoroughly)

Acidification and Neutralization of Samples and Standards

1. 200 µL Standards or **prediluted** Sample added into Reaction Caps (Eppendorf-Caps).
2. 20 µL 1 N HCl added to all caps
3. mixed thoroughly (vortex) and let stand for 15 minutes
4. 20 µL 1N NaOH added for neutralization and mixed thoroughly.
5. After neutralization the sample had a pH value between 7 and 8.

Test Procedure

Instrument used- Triturus automated ELISA machine

1. the Microtiter wells were set in the frame holder.

2. **100 µL** of each pretreated ***Standard, Control*** and **samples dispensed** with new disposable tips into appropriate wells.

3. plates covered and incubated for 3 hours incubation at room temperature.

4. the wells were Rinsed **3 times** with diluted wash solution, 300 µl per Well. Washing performed with the washer.

5. **100 µL** *Antiserum* Dispensed into all wells.

6. Incubated for **120 minutes** at room temperature.

7. the wells Rinsed **3 times** with diluted wash solution, 300 µl per Well.

8. **100 µl** *Enzyme Conjugate* (Anti Mouse Biotin) Dispensed into each well.

9. Incubated **45 minutes** at room temperature.

10. the wells Rinsed **3 times** with diluted wash solution, 300 µl per Well.

11. **100 µl** *Enzyme Complex* Dispensed into each well.

12. Incubated **45 minutes** at room temperature.

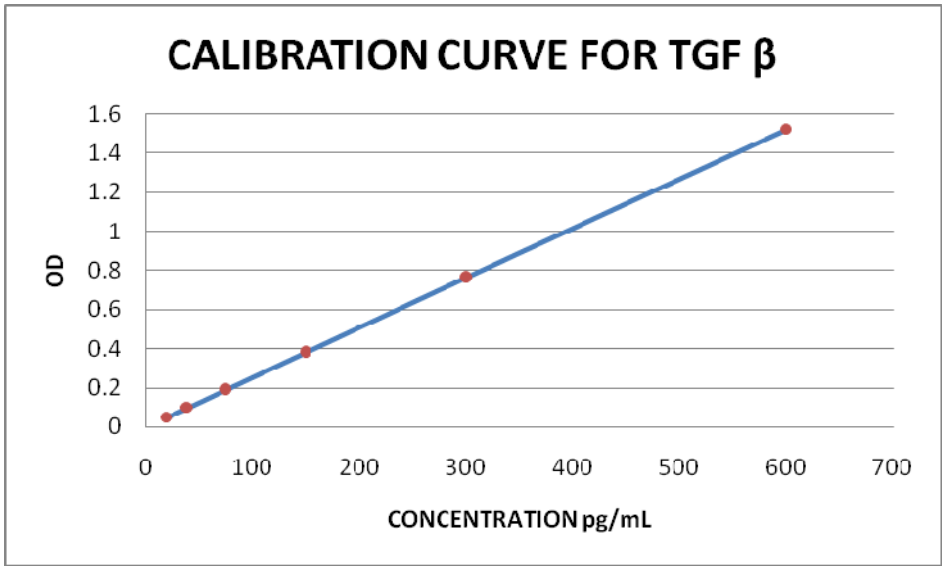
13 the wells Rinsed **3 times** with diluted wash solution, 300 µl per Well.

14. **100 µL** of ***Substrate Solution*** Added to each well.

15. Incubated for **15 minutes** at room temperature.

16. the enzymatic reaction Stopped by adding **50 µL** of ***Stop Solution*** to each well.

17. the absorbance (OD) of each well Determined at **450 ± 10 nm** with a microtiter plate reader, within 10 minutes of adding the stop solution.



Calculation of Results

1. the average absorbance values for each set of standards, controls and patient samples were Calculated.

2. Using semi-logarithmic graph paper, a standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample the corresponding concentration was determined from the standard curve.

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

The statistical software SPSS pc+ (Statistical Package for Social Science) was used for statistical analysis.

Mean and Standard deviation were estimated from the sample each study group. The mean values were compared by students' t-test to calculate the p value. P value < 0.005 was considered significant.

Chi square test was used for comparison of the variables between controls and cases.

Scatter diagrams were plotted to show the relationship between cases and controls on the levels of Lp(a) and TGF-β

Pearson's Correlation analysis was used to correlate the levels of Lp(a) and TGF-β.

Charts were plotted for the significant lipid parameters to highlight their levels in cases and controls.

RESULTS

TABLE-I : MASTER CHART CASES

S.No	AGE yrs	SEX	SMK	ALC	DM	BMI kg/m^2	Tot CHOL mg/dL	TGL mg/dL	HDL mg/dL	LDL mg/dL	Lp(a) mg/dL	TGF-β pg/mL
1	35	F	NO	NO	NO	18.8	156	148	38	88.4	34.9	264
2	40	M	NO	YES	NO	23.7	188	156	40	116.8	31.1	203
3	41	M	YES	YES	NO	25.2	181	148	41.5	109.9	37.9	185
4	36	M	NO	NO	NO	27.2	253	131	44	182.8	38.5	165
5	38	M	YES	YES	NO	28.6	238	157	36.5	170.1	36.9	200
6	51	M	YES	YES	YES	32.5	156	169	30	92.2	73.4	136
7	47	M	YES	YES	NO	20.3	197	189	25.8	133.4	53.6	174
8	58	M	YES	YES	NO	32.4	293	194	31.2	223	75.9	146
9	40	M	YES	YES	NO	34.6	286	253	36	199.4	84.7	120
10	60	M	NO	YES	NO	28.3	230	295	28.5	142.5	82.7	128
11	37	M	YES	YES	NO	21.8	215	186	31.0	146.8	58.5	342
12	40	M	YES	YES	NO	27.9	176	185	34.3	104.7	60.8	179
13	41	M	YES	YES	NO	28.2	200	158	41	127.4	55.1	156
14	41	M	YES	YES	NO	29.8	250	175	39	177.4	40.3	214
15	72	M	YES	YES	NO	25.3	245	252	35.6	159	85.7	108
16	45	M	YES	YES	YES	34.5	310	271	32.0	223.8	75.4	138
17	57	M	YES	YES	YES	26.0	186	220	41	101	71.6	196
18	62	M	YES	YES	NO	19.6	135	152	32.5	72.1	74.5	208
19	52	M	YES	YES	YES	27.4	278	253	33	194.4	89.2	124
20	50	F	NO	NO	YES	18.8	136	125	42	69	30	286
21	43	M	YES	YES	NO	22.5	120	165	41	46	37.2	242
22	55	M	YES	NO	NO	28.6	200	268	36	110.4	87.1	140
23	54	M	YES	YES	NO	29.1	186	235	40.5	98.5	51.3	200
24	41	M	YES	YES	NO	25.4	186	135	38	121	23.6	346
25	50	M	YES	YES	NO	20.2	146	102	32.5	93.1	65	210

S.No	AGE yrs	SEX	SMK	ALC	DM	BMI kg/m^2	Tot CHOL mg/dL	TGL mg/dL	HDL mg/dL	LDL mg/dL	Lp(a) mg/dL	TGF-β pg/mL
26	53	M	YES	YES	YES	29.7	230	196	37.6	153.2	53.5	184
27	75	M	NO	NO	YES	23.0	200	245	40.5	110.5	70.3	170
28	55	M	YES	YES	NO	25.9	187	168	44	109.4	41.2	179
29	49	M	YES	YES	YES	25.1	217	184	41	139.2	47.4	168
30	45	M	YES	YES	NO	25.0	154	163	37	84.4	53.6	251
31	65	F	NO	NO	YES	18.8	142	124	45	72.2	33.5	278
32	65	M	NO	YES	YES	25.3	184	156	42	110.8	71.6	144
33	57	M	YES	YES	NO	28.7	210	185	38	135	85.7	106
34	49	M	YES	YES	YES	21.2	234	110	47.5	164.5	33	434
35	61	M	YES	YES	NO	27.8	156	182	39.2	80.4	72.3	156
36	54	M	YES	YES	YES	27.2	200	225	41.2	113.8	59.6	203
37	40	M	YES	YES	NO	26.4	182	231	43.4	92.4	29.3	255
38	60	M	YES	YES	YES	29.6	258	197	36.7	181.9	78.8	165
39	49	M	YES	YES	YES	29.3	236	262	38.6	145	88.5	122
40	56	M	YES	YES	NO	23.5	142	188	40.6	63.8	83	137
41	45	M	YES	YES	NO	26.0	150	148	32	88.4	86.9	132
42	52	M	YES	YES	YES	21.5	132	168	30	68.4	87.7	110
43	47	M	YES	YES	YES	25.4	190	178	37.5	116.9	86.2	108
44	60	M	YES	YES	YES	18.4	176	245	38	89	87.4	142
45	52	M	YES	YES	NO	27.5	260	215	41.3	175.7	73.5	175
46	70	M	YES	YES	YES	25.8	196	268	40.4	102	85.4	136
47	63	M	YES	YES	YES	26.3	148	256	35.6	61.2	71.6	158
48	53	M	NO	NO	YES	23.0	126	143	39.7	57.7	72.5	156
49	70	M	YES	YES	NO	25.7	146	198	37	69.4	86	128
50	68	M	NO	YES	NO	21.9	154	146	28.8	96	70.1	134

TABLE- II : MASTER CHART CONTROLS

S.No	AGE yrs	SEX	SMK	ALC	BMI (kg/m^2)	T.CHOL (mg/dL)	TGL (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Lp(a) (mg/dL)	TGF-β (pg/mL)
1	45	M	NO	NO	23.7	150	102	42.3	87.3	8.9	376
2	60	M	NO	NO	23.5	110	115	40	47	12.0	410
3	60	M	NO	YES	22.0	190	152	41.5	118.1	11.7	516
4	40	M	NO	YES	23.5	162	125	50	87	8.2	569
5	50	F	NO	NO	20.4	128	95	47	62	8.5	440
6	65	M	NO	NO	22.1	148	142	52.5	67.1	13.3	477
7	36	F	NO	NO	24.2	125	106	47.6	56.2	9.9	380
8	45	M	YES	YES	22.9	165	127	41.6	98	12.6	302
9	55	M	YES	NO	25.1	146	132	50	69.6	11.6	325
10	68	M	NO	NO	20.2	150	100	45.7	84.3	10.7	372
11	75	M	NO	YES	23.0	156	127	42	88.6	17.5	565
12	56	M	YES	YES	25.7	148	121	41.7	82.1	17.9	565
13	72	M	NO	NO	32.1	162	120	42.3	95.7	18.9	446
14	45	M	NO	YES	23.7	148	111	45	80.8	8.5	534
15	49	M	NO	NO	23.5	151	96	43	88.8	10.7	457
16	50	M	YES	YES	23.0	162	105	41.6	99.4	10.8	476
17	66	F	NO	NO	21.0	145	97	46.8	78.8	9.2	451
18	51	M	NO	NO	23.6	145	98	48	77.4	10.2	468
19	62	M	NO	NO	22.1	165	128	40.7	98.7	10.8	375
20	52	M	YES	YES	23.6	146	135	49.2	69.8	9.6	527
21	49	M	NO	NO	22.7	175	108	45.4	108	12.2	434
22	47	M	NO	NO	25.2	183	125	43.5	114.5	9.9	560
23	52	M	NO	NO	22.2	163	117	44	95.6	10.9	493
24	40	M	YES	NO	23.0	153	97	47	86.6	9.5	418
25	40	M	YES	YES	25.1	188	119	44.3	119.9	10.2	439

S.No	AGE yrs	SEX	SMK	ALC	BMI (kg/m^2)	T.CHOL (mg/dL)	TGL (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Lp(a) (mg/dL)	TGF-β (pg/mL)
26	49	M	NO	NO	25.0	175	102	43.6	111	9.5	514
27	41	M	YES	NO	23.5	159	121	47.2	87.6	8.9	394
28	53	M	NO	NO	25.4	149	119	45	80.2	10.5	491
29	40	M	NO	NO	24.9	168	97	45.2	103.4	7.1	585
30	47	M	NO	NO	23.3	154	123	42.7	86.7	10.2	529
31	42	M	NO	NO	23.2	147	103	41.8	84.6	10.1	352
32	57	M	NO	NO	22.9	173	121	38.6	110.2	12.15	420
33	60	M	YES	YES	21.8	169	121	38	106.8	20.5	380
34	62	M	NO	NO	23.7	157	118	40	93.4	18.1	294
35	62	M	NO	NO	22.7	148	121	42.5	81.3	18.7	326
36	37	M	YES	NO	25.1	193	101	47.6	125.2	9.6	572
37	70	M	NO	NO	19.5	170	116	44.2	102.6	21.5	542
38	53	M	YES	NO	19.7	148	92	43.5	86.9	11.7	511
39	54	M	YES	YES	23.1	173	106	42	109.8	12.9	438
40	51	M	NO	NO	26.1	192	126	47.3	119.3	12.6	394
41	38	M	NO	NO	23.7	145	86	53.5	74.3	10.2	494
42	41	M	YES	YES	22.8	125	143	47.2	49.2	13.0	486
43	58	M	NO	NO	23.0	155	131	43.8	85	13.4	476
44	70	M	NO	NO	20.8	173	48	41.7	111.7	15.6	346
45	38	M	NO	YES	22.7	136	82	50.6	69	9.9	642
46	43	M	NO	NO	23.0	160	105	44	95	12.0	573
47	41	M	YES	YES	25.5	168	137	44.2	96.4	14.7	537
48	54	M	NO	YES	24.5	183	127	42.0	115.6	13.8	570
49	55	M	NO	NO	22.8	140	106	44	74.8	13.2	565
50	52	M	NO	NO	23.0	152	108	46.7	83.7	12.6	583

TABLE- III : CHARACTERISTICS OF PATIENTS WITH PAD AND OF CONTROLS

	Group	N	Mean	Std. Deviation	p value
Age in years	Control	50	51.96	10.12	0.992
	Cases	50	51.98	10.23	
BMI	Control	50	23.37	1.96	<0.001**
	Cases	50	25.69	3.97	
Total Chol	Control	50	157.52	17.72	<0.001**
	Cases	50	195.14	47.25	
TGL	Control	50	113.20	17.96	<0.001**
	Cases	50	190.06	47.72	
HDL	Control	50	44.59	3.40	<0.001**
	Cases	50	37.47	4.75	
LDL	Control	50	90.10	18.38	<0.001**
	Cases	50	119.68	44.28	
Lp(a)	Control	50	12.13	3.32	<0.001**
	Cases	50	63.27	20.52	
TGF	Control	50	467.78	85.60	<0.001**
	Cases	50	182.82	67.30	

** denotes significance at 1% level (highly significant)

TABLE IV : COMPARISON OF LEVELS OF Lp(a) AND TGF-β AMONG DIABETICS & NON DIABETICS

	DM	Mean	Std. Deviation	P value
Lp(a)	Yes	68.33	19.32	0.157 (NS)
	No	59.89	20.92	
TGF	Yes	177.9	76.96	0.667 (NS)
	No	186.1	61.18	

NS – Not significant at 5% level.

TABLE Va : Correlation between Lp(a) and TGF-β - CASES

		Lp(a)	TGF
Lp(a)	Pearson Correlation	1	-.750(**)
	Sig. (2-tailed)	.	.000
	N	50	50
TGF	Pearson Correlation	-.750(**)	1
	Sig. (2-tailed)	.000	.
	N	50	50

** Correlation is significant at the 0.01 level (2-tailed).

TABLE Vb : Correlation between Lp(a) and TGF-β - CONTROLS

		Lp(a)	TGF
Lp(a)	Pearson Correlation	1	-.150
	Sig. (2-tailed)	.	.299
	N	50	50
TGF	Pearson Correlation	-.150	1
	Sig. (2-tailed)	.299	.
	N	50	50

TABLE VIa : Correlation between other lipid parameters with Lp(a) and TGF-β - CASES

		Lp(a)	TGF- β
Total Cholesterol	Pearson Correlation	.131	-.143
	Sig. (2-tailed)	.363	.323
	N	50	50
TGL	Pearson Correlation	.552(**)	-.478(**)
	Sig. (2-tailed)	.000	.000
	N	50	50
HDL	Pearson Correlation	-.468(**)	.363(**)
	Sig. (2-tailed)	.001	.010
	N	50	50
LDL	Pearson Correlation	.071	-.088
	Sig. (2-tailed)	.626	.544
	N	50	50

** Correlation is significant at the 0.01 level (2-tailed).

TABLE VIb : Correlation between other lipid parameters with Lp(a) and TGF-β - CONTROLS

		Lp(a)	TGF- β
Total Cholesterol	Pearson Correlation	.116	.092
	Sig. (2-tailed)	.423	.524
	N	50	50
TGL	Pearson Correlation	.213	.032
	Sig. (2-tailed)	.137	.827
	N	50	50
HDL	Pearson Correlation	-.433(**)	.262
	Sig. (2-tailed)	.002	.066
	N	50	50
LDL	Pearson Correlation	.138	.051
	Sig. (2-tailed)	.338	.727
	N	50	50

** Correlation is significant at the 0.01 level (2-tailed).

FIGURE 1 : MEAN BMI & LIPID PROFILE IN CASES AND CONTROLS

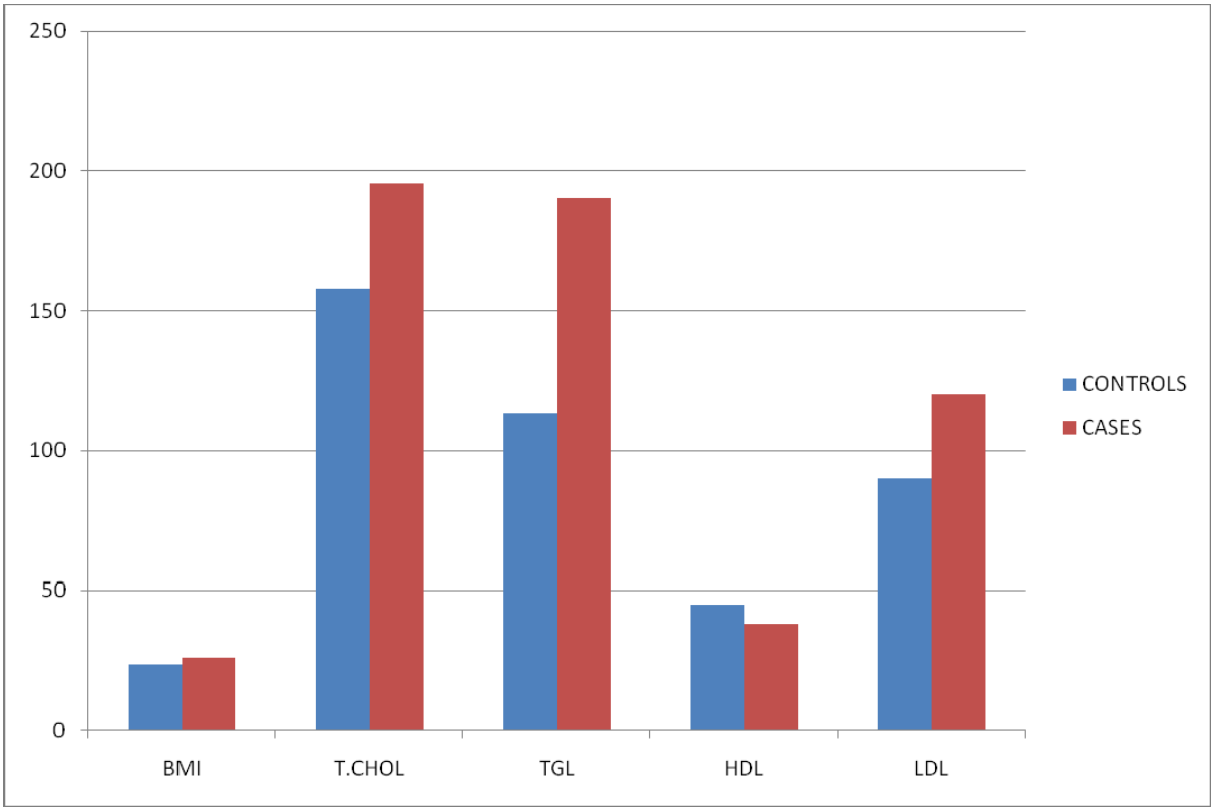


FIGURE 2 : MEAN Lp(a) AND TGF- β IN CASES AND CASES

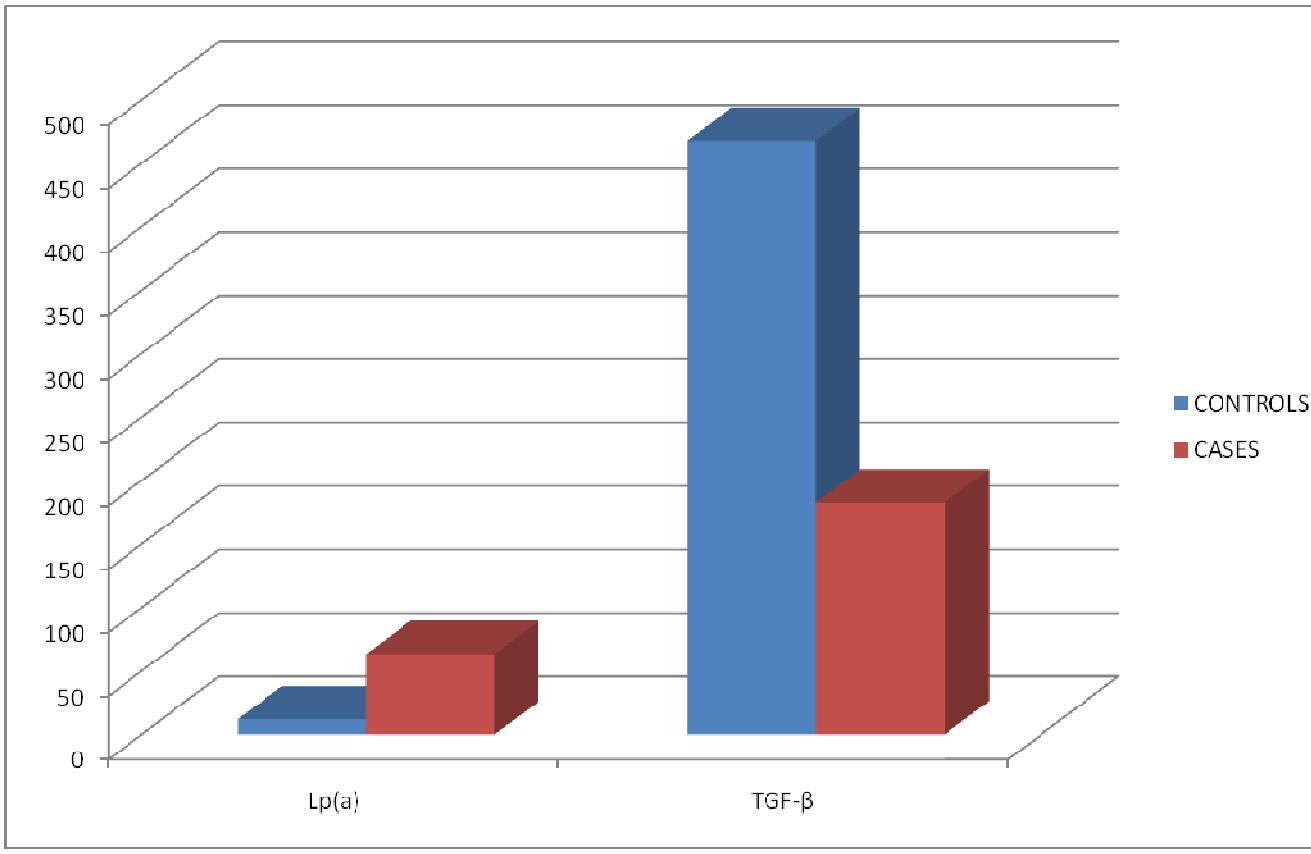


FIGURE 3 : Lp(a) LEVELS AMONG CASES AND CONTROLS

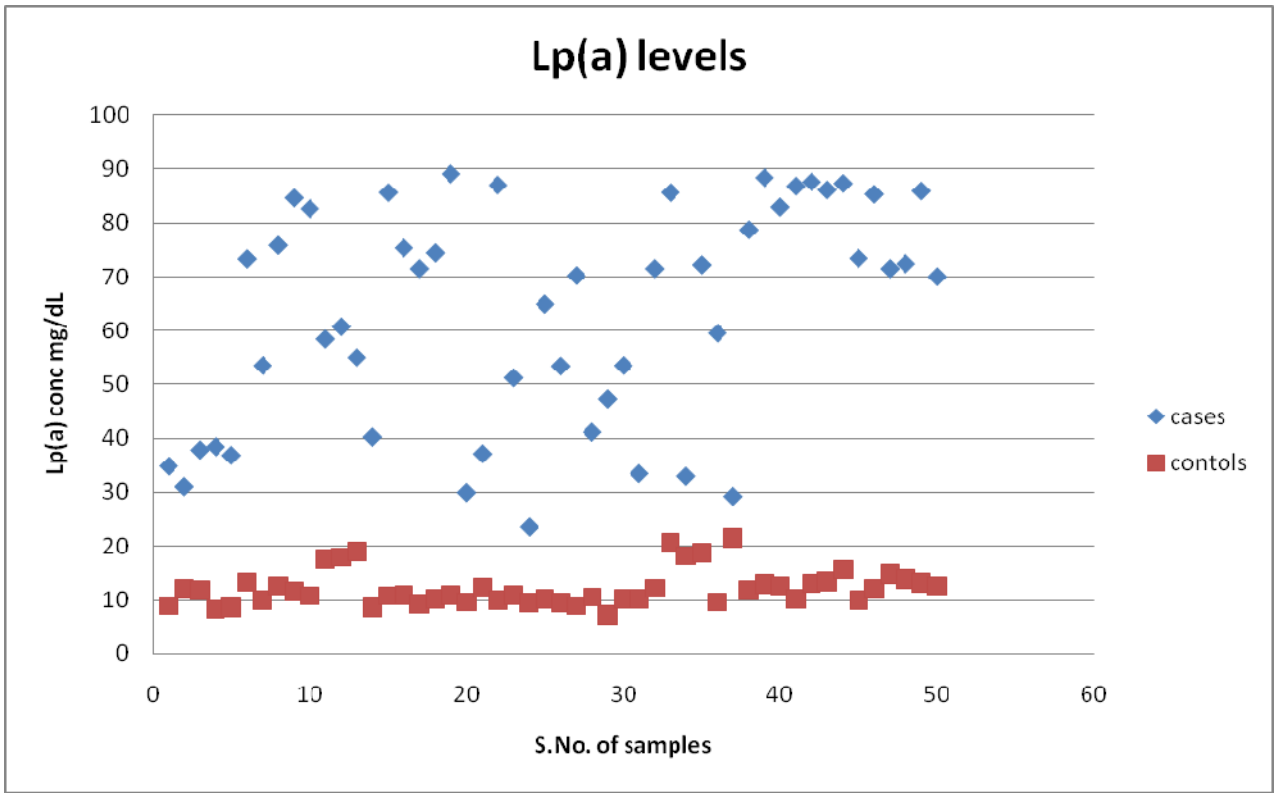


FIGURE 4 : TGF- β LEVELS AMONG CASES AND CONTROLS

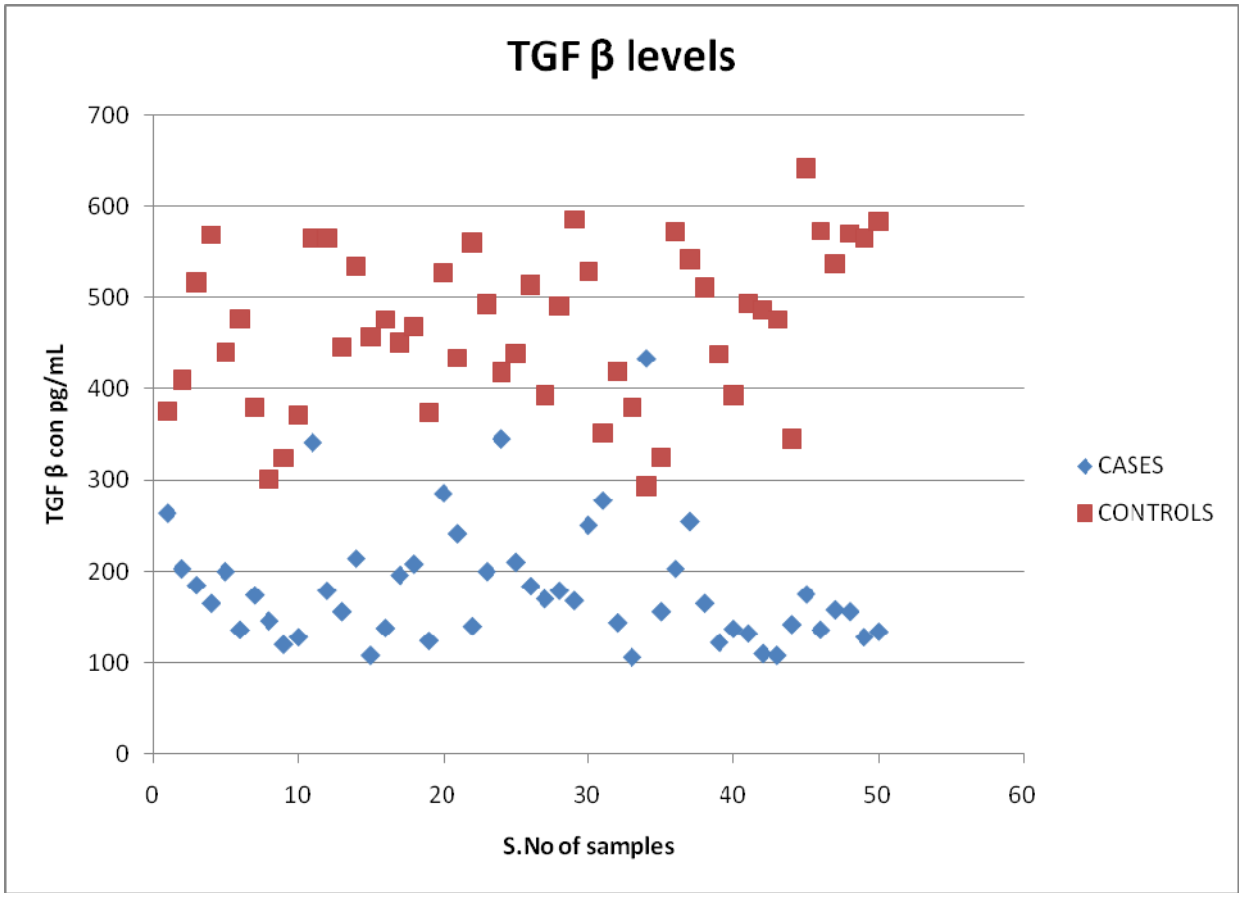


FIGURE 5 : MEAN BMI FOR CASES AND CONTROLS

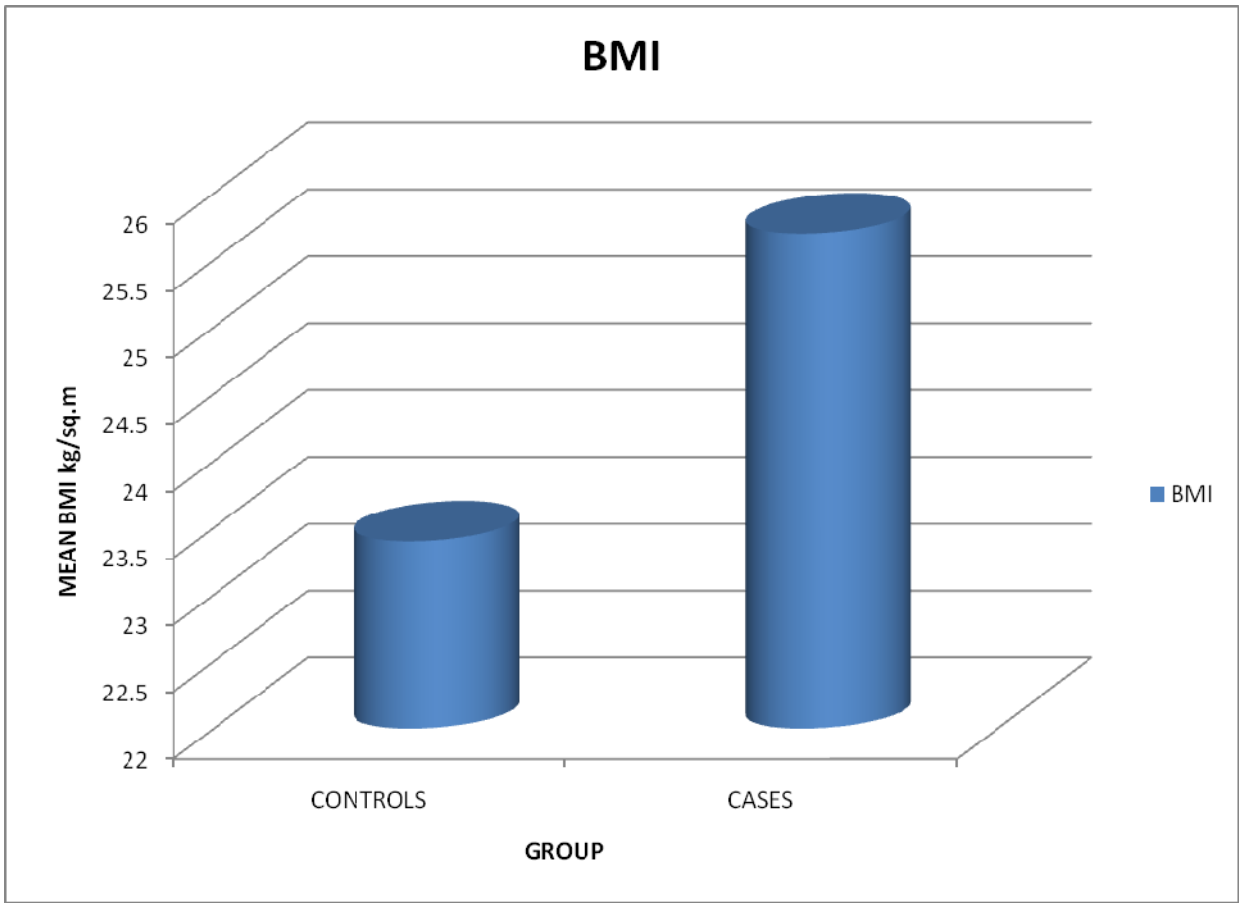


FIGURE 6 : MEAN TOTAL CHOLESTEROL FOR CASES AND CONTROLS

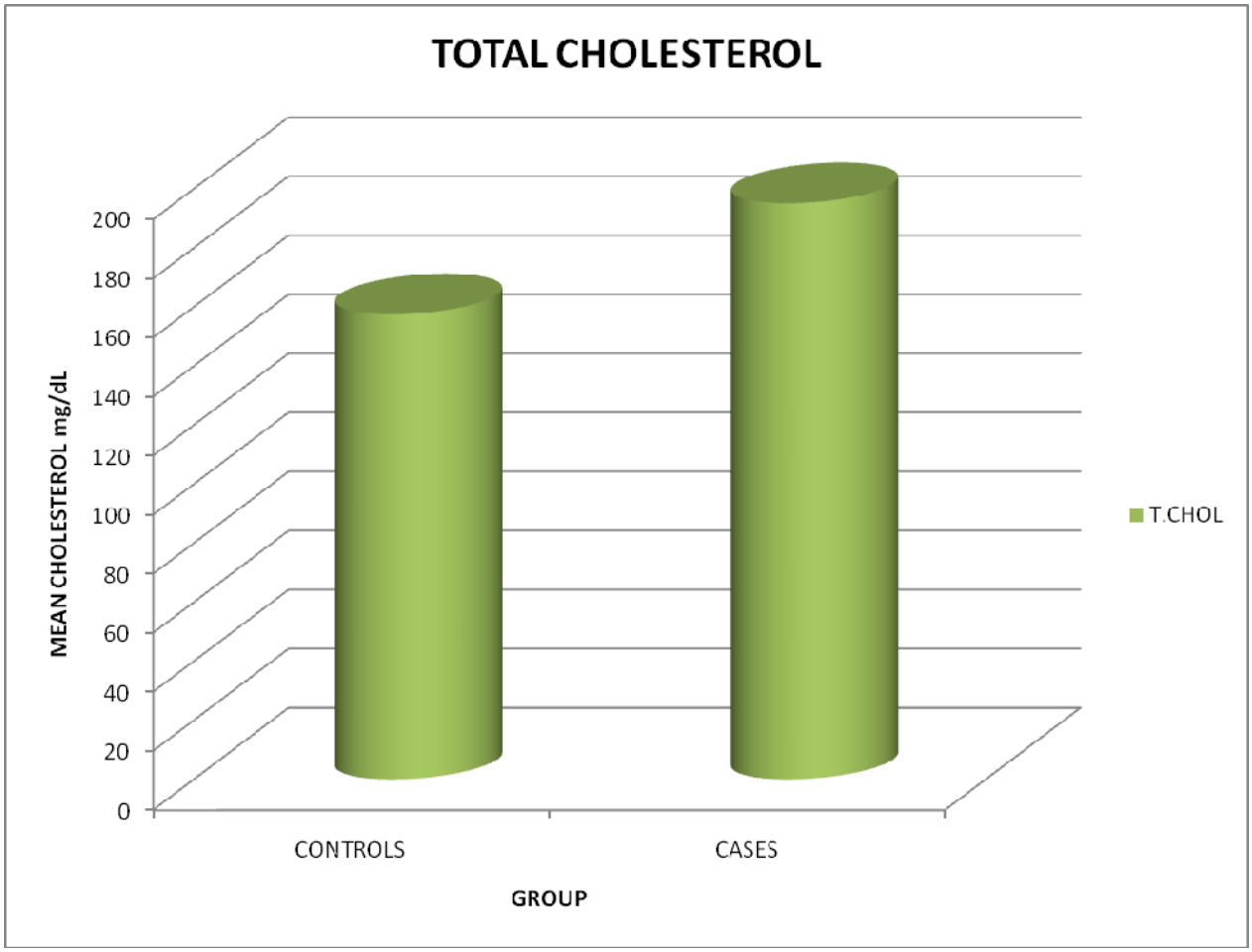


FIGURE 7 : MEAN TGL FOR CASES AND CONTROLS

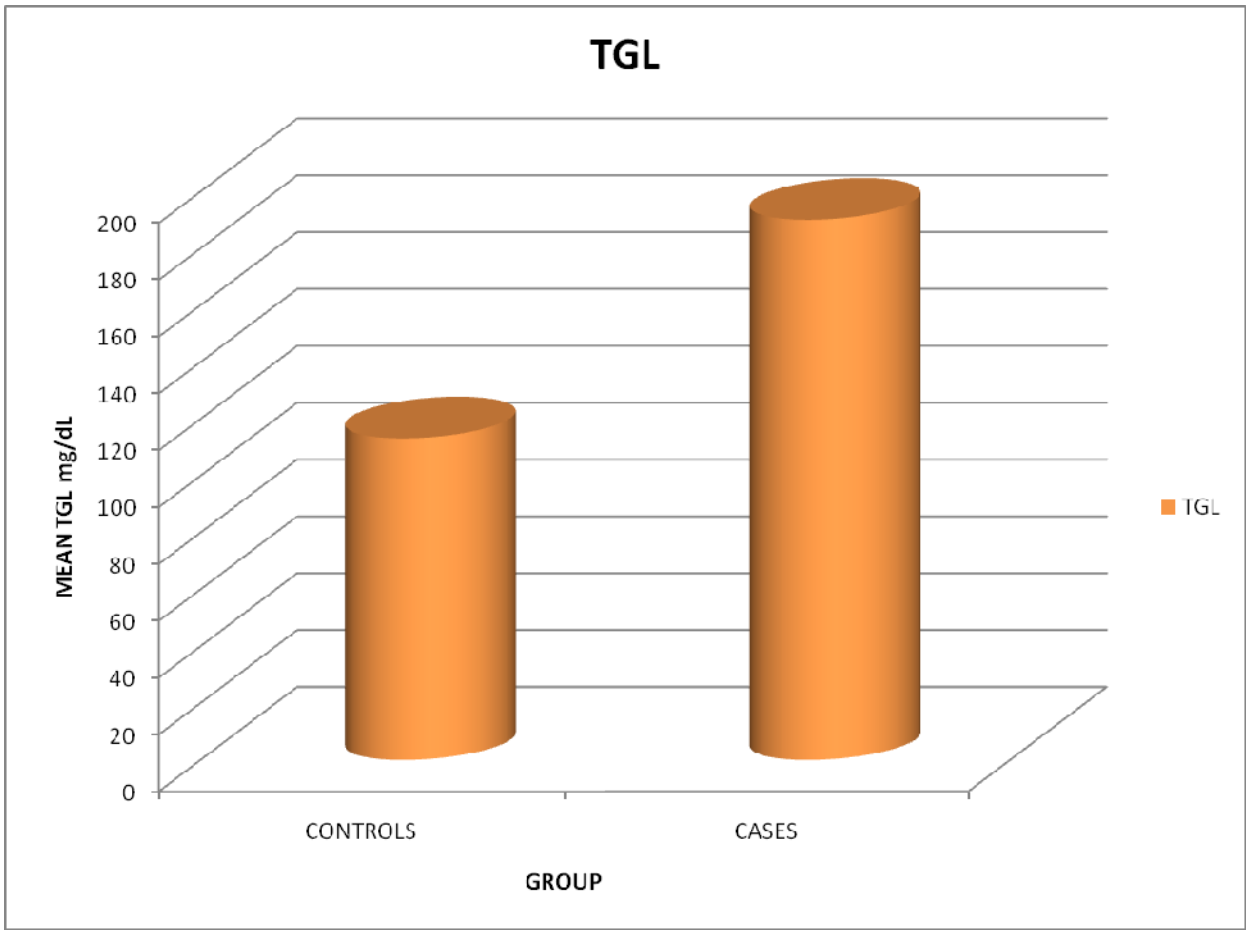


FIGURE 8 : MEAN LDL FOR CASES AND CONTROLS

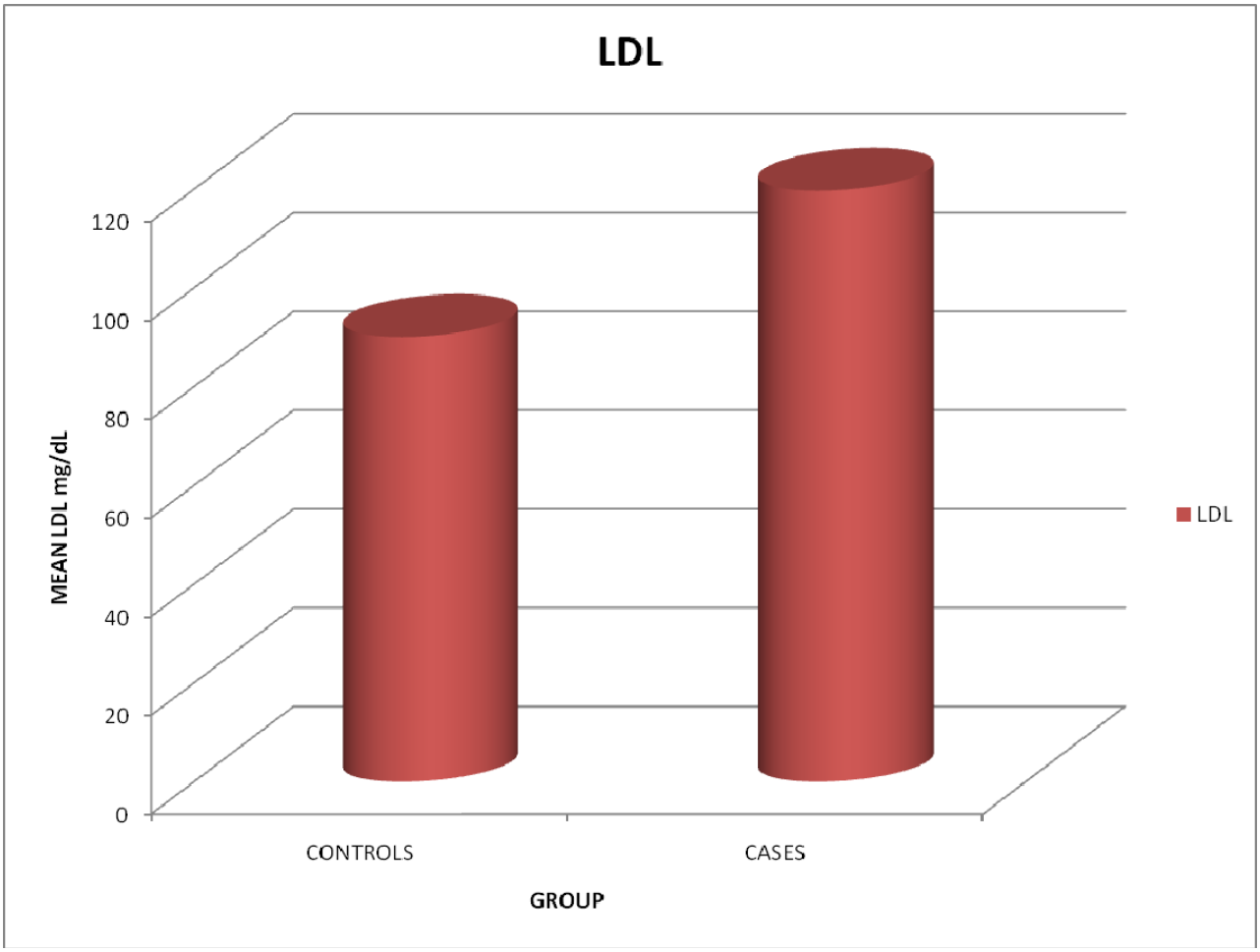


FIGURE 9 : MEAN HDL FOR CASES AND CONTROLS

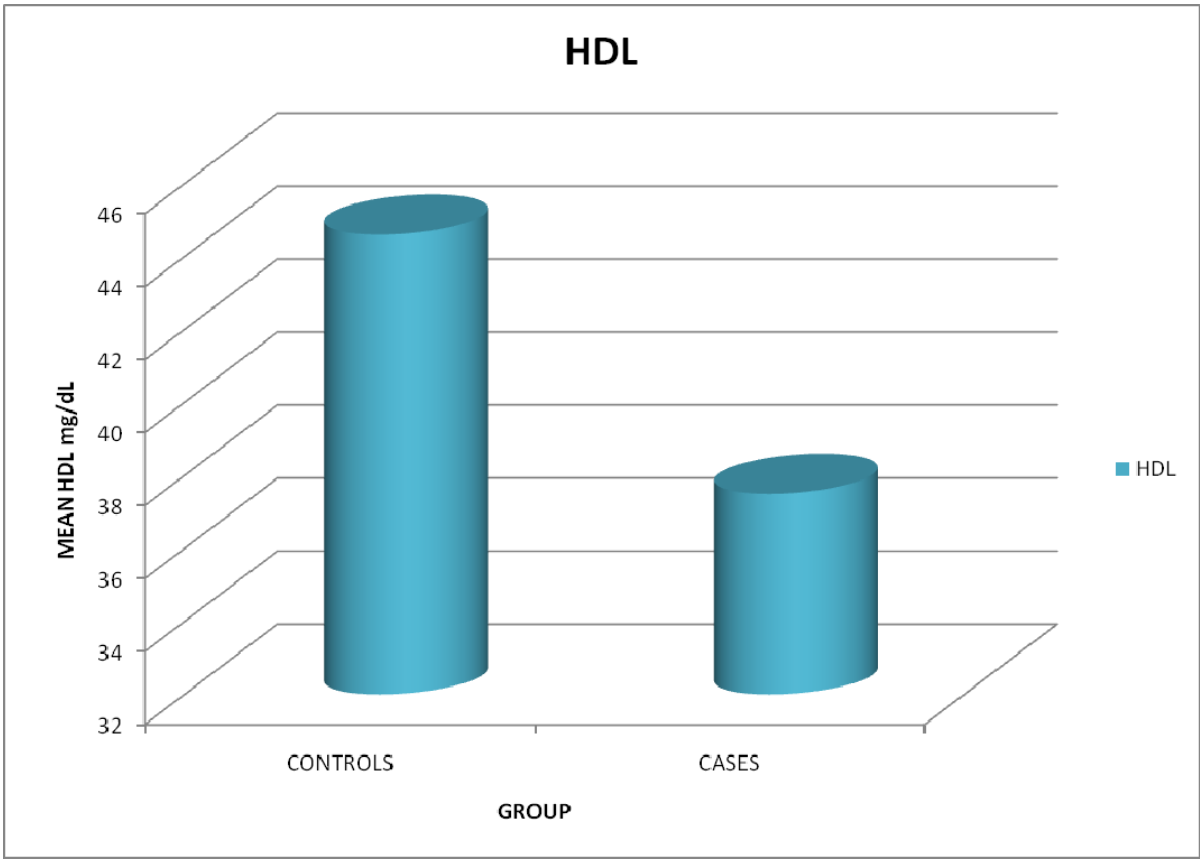


FIGURE 10 : MEAN Lp(a) FOR CASES AND CONTROLS

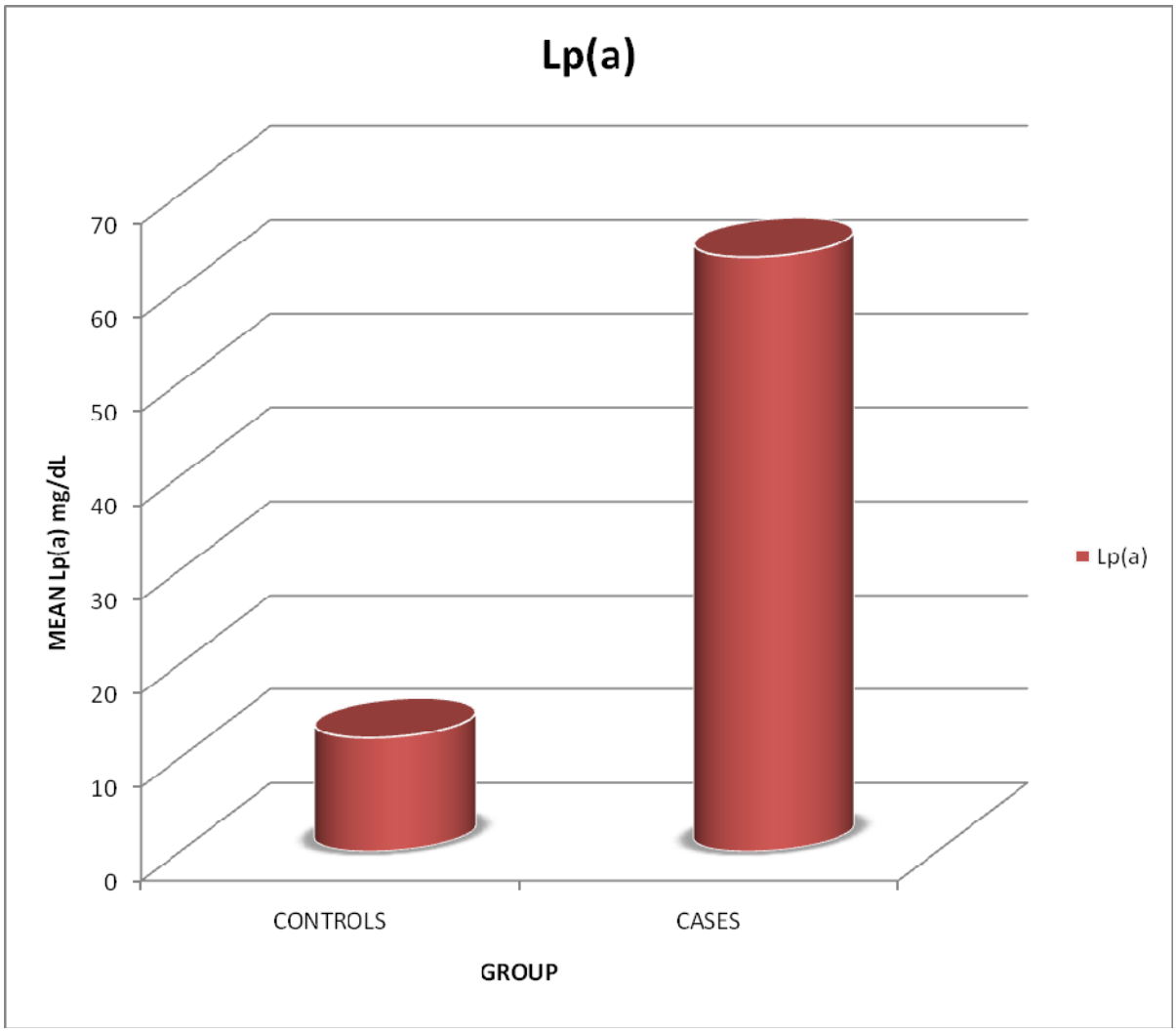
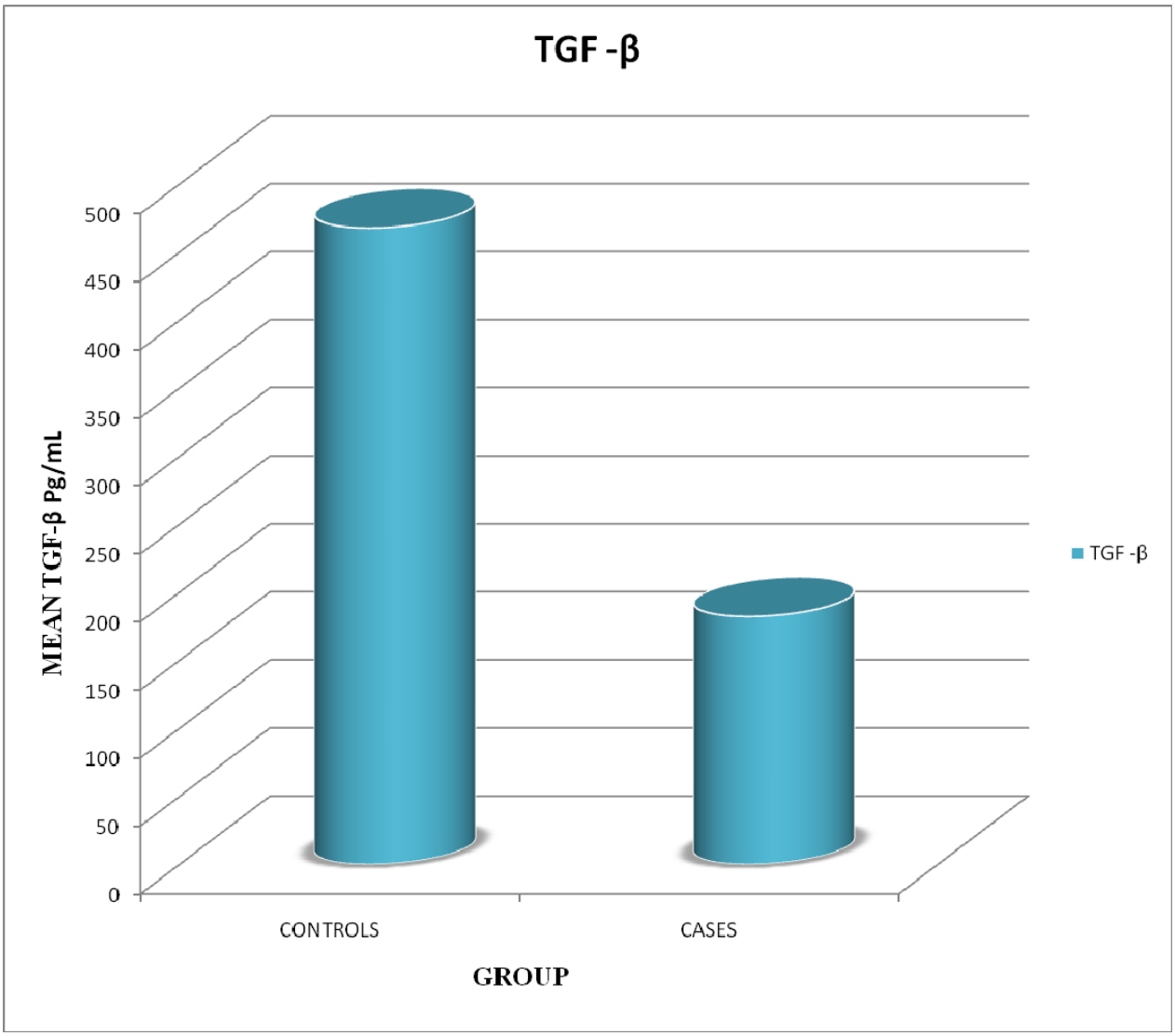


FIGURE 11 : MEAN TGF-β FOR CASES AND CONTROLS



RESULTS

The age, sex, Body Mass Index, serum lipid levels, Lp(a) and TGF-β levels of the 50 patients of Atherosclerotic peripheral Vascular disease are listed in Table I.

The age, sex, Body Mass Index, serum lipid levels, Lp(a) and TGF-β levels of the 50 healthy controls are listed in Table II.

The mean values of the studied parameters of the patients were compared with that of the controls using students' independent t-test in Table III. P value <0.05 was taken as significant.

Mean BMI for cases with PVD (25.6 +/- 3.9) is significantly higher than the mean BMI for controls (23.3 +/- 1.9). ($p < 0.01$)

Mean serum total cholesterol level in cases (157.5 +/- 17.7) is significantly higher than that of the controls (195.1 +/- 47.2) ($p < 0.01$)

Mean serum Triglycerides in cases (190.1 +/- 47.7) is significantly higher than that of controls (113.2 +/- 17.9) ($p < 0.01$)

Mean serum LDL in cases (119.7 +/- 44.2) is significantly higher than that of controls (90.1 +/- 18.3) ($p < 0.01$)

Mean serum HDL in cases (37.4 +/- 4.7) is significantly lower than that of controls (44.5 +/- 3.4) ($p < 0.01$)

Mean serum Lp(a) in cases (63.2 +/- 20.5) is significantly higher than that of controls (12.1 +/- 3.3) ($p < 0.01$). Mean serum TGF-β in cases (182.8 +/- 67.3) is significantly lower than that of controls (467.7 +/- 85.6) ($p < 0.01$)

Thus increased BMI, total cholesterol, Triglyceride, Low Density Lipoprotein, decreased High density lipoprotein, increased Lipoprotein (a) and decreased TGF- β are significant contributing factors for the occurrence of Atherosclerotic peripheral vascular disease.

Since Diabetes mellitus is a single most important factor that can contribute to both atherosclerotic PVD and increase in Lp(a), the means of the level of Lp(a) in patients with and without DM were compared in Table IV. There was no significant difference in the level of elevation of Lp(a) in these two groups so it can be inferred that Lp(a) contributes independently to the occurrence of PVD.

Tables Va and Vb show The relationship between the levels of Lp(a) and TGF-β using the correlation analysis, in cases and controls, respectively. There was no statistically significant correlation between Lp(a) and TGF-β in control subjects but we found a strongly negative correlation between these two parameters in cases of PVD.

Table VIa and VIb show the correlation between the levels of the lipid parameters Total Cholesterol, Triglyceride, HDL and LDL with Lp(a) and TGF- β among cases and controls, respectively. There is a statistically significant positive correlation with TGL levels and Lp(a), negative correlation with HDL and Lp(a), positive correlation with HDL and TGF- β, negative correlation with TGL and TGF- β, and this is among cases. The only significant correlation found among controls is between HDL and Lp(a), a negative one.Figures 1 through 11 show the mean levels of various parameters viz. BMI, Total Cholesterol, Triglyceride, HDL, LDL, Lipoprotein (a) and TGF- β in cases and controls. Figures 3 and 4 depict the distribution of the levels of Lp(a) and TGF- β among cases and controls by means of scatter diagram.

DISCUSSION

DISCUSSION

Peripheral Vascular disease is an important health care problem in the developed countries[117] and the concern is constantly increasing in the developing nations. Extensive research has been conducted on the prevalence, risk factors and patient characteristics in western countries, but very few studies have been done in India. The general prevalence of atherosclerotic PVD is found to be higher in western countries than South Asian population. There are differences in the risk factors based on ethnicity, as shown in many of the studies.

The incidence of atherosclerotic PVD is on the rise rampantly and exponentially in India due to the following reasons: sheer increase in population size due to natural growth, ageing of the population which makes them more vulnerable to chronic diseases (advanced age is an important risk factor for atherosclerosis and PVD), increased vulnerability due to life style changes that promote PVD, India becoming the Diabetic capital of the world (the occurrence of atherosclerotic complications is several times more pronounced and severe in diabetics).

S.A.V.E (Stroke, Aneurysm and Vascular Evaluation) India project is a study aimed to determine the incidence and prevalence of Stroke, Aneurysms and Occlusive Peripheral Vascular Disease (PVD) in the Indian population, to determine atherosclerotic and non-atherosclerotic risk factors , to assess appropriateness of utilization of diagnostic imaging in the screening and to diagnose the same in a timely fashion for early treatment of life and limb threatening vascular conditions.

This study has attempted to determine the characteristics of atherosclerotic PVD and the risk factors in a south Indian population, the subjects mainly belonging to tamilnadu, some of them having origin in Andhra Pradesh. But there did not seem to be any gross difference in the measured parameters between them, so they are evaluated as a single group of cases.

The gender distribution of cases in this present study is 47 males and 3 females, 2 of the females in the post menopausal age group. This clearly shows that male sex is in itself a risk factor for atherosclerotic PVD, estrogen being protective in women till menopause.

The conventional risk factors like increased BMI, total cholesterol, triglycerides and LDL and decreased HDL are significantly higher in the cases, emphasizing their causative role once again. Hypertriglyceridemia seems to be more common than hypercholesterolemia, the TGL levels on the upper level even in the controls. Also, HDL cholesterol level was on the lower limit in cases and just adequately normal in most of the controls.

This study has given importance to evaluate the association of lipoprotein(a) in atherosclerotic PVD and its relation to an essential antiatherogenic factor, TGF-β. While the role of TGF- β in atherosclerosis has been studied extensively, there have been few studies relating to atherosclerotic PVD. The relationship between Lp(a) and TGF- β has been described since years ago, that Lp(a) affects the activity of TGF β adversely. This study is the first to evaluate this relationship in atherosclerotic PVD in a south Indian population. Since TGF β has shown to be associated independently with hypertension, and the pathogenesis of hypertension is both interlinked and many a times confounding with atherosclerosis, it was decided to exclude systemic hypertension from the study.

The levels of Lp(a) are increased to enormous proportions in cases than controls, and the TGF levels are decreased in the same. The distribution of TGF levels is wide as seen in the scatter diagram, both in cases and controls. There is negative correlation between Lp(a) and TGF β only in cases and not in controls.

This is because of the extensive cytokine interactions in atherosclerosis and in normal individuals as such. Lp(a) is not the only factor that affects TGF beta, but it definitely affects its

adversely when present in excess. The level of Lp(a) is in turn determined by the genetic arrangement of apo(a) gene. The level of Lp(a) is inversely proportional to the number of kringle repeats in the apo(a) gene.

Lp(a) shows positive correlation with TGL and not total cholesterol or LDL in cases, and there is no significant correlation in controls, possibly due to the genetic makeup of the cases. Negative correlation is seen with HDL both in cases and controls, proving that HDL is protective. TGF beta has no association with any of the lipid parameters in controls but has negative correlation with TGL and significant positive correlation with HDL in cases.

This study has shown that Lp(a) is a causative factor for atherosclerotic PVD. The fact is not forgotten that the rise in its levels is also an effect of other etiological parameters like smoking, alcoholism and especially, Diabetes Mellitus. Its levels were analyzed between diabetics and non diabetics, a slight increase in mean levels was noted in diabetics, but still statistically not significant. So Lp(a) can be considered an independent risk factor for atherosclerotic peripheral vascular disease.

In addition to its well established mechanisms of pathogenesis, atherogenicity due to the constituent LDL particle and thrombogenicity due to the apo(a) moiety which has structural analogy with plasminogen and so inhibits fibrinolysis, the theory that Lp(a) stimulates smooth muscle proliferation by inhibiting the protective TGF beta, has been well substantiated in this study.

This could open up a wide arena for research in order to answer the following unanswered questions in this study:

- Why is there wide variation in the levels of TGF beta among apparently healthy individuals?
- Why isn't there a consistent proportion of decrease of TGF beta with increase in Lp(a)?
- Does the genetic arrangement of Lp(a) affect the gene expression of TGF beta? If so how?
- What are the other factors that play intermediary role between the various lipid parameters and TGF beta?

The knowledge of the answers to these unanswered questions can help in making atherosclerotic PVD more amenable to treatment and provide better quality of life for the affected.

CONCLUSION

CONCLUSION

From the results of the study it can be concluded that,

Increased level of Lipoprotein (a) and decreased level of TGF-β are independent risk factors for atherosclerotic peripheral vascular disease, along with the other established risk factors – Smoking, Alcoholism, Diabetes mellitus, Increased body mass index, Increased total Cholesterol, Increased Triglyceride, Increased Low Density Lipoprotein and Decreased High Density lipoprotein.

Lipoprotein (a) inhibits TGF-β and decreases its level in the blood, the inhibition more pronounced at higher levels of Lp(a).

Hypertriglyceridemia is a more significant risk factor than hypercholesterolemia.

High Density Lipoprotein is protective against atherosclerotic peripheral vascular disease.

Estimation of Lipoprotein (a) can be included in the routine analysis of lipid profile in patients with atherosclerotic peripheral vascular disease.

FUTURE PROSPECTS OF THE STUDY

FUTURE PROSPECTS OF THE STUDY

a. Studies on a larger scale can be undertaken in different parts of India and Asia to establish definite cut off values for Lp(a) and TGF-β according to the ethnicity and lifestyle.
b. Genetic studies focusing on the gene pattern of lipoprotein (a), the apo A gene, and its influence over the levels of serum Lp(a) and relationship with TGF-β, can be encouraged since it will give a clearer insight into the pathogenic relationship between them.
c. Research aimed at developing drugs against Lipoprotein (a) and its pathologic consequences can be done.
d. Further research works focused on identifying strategies to improve the TGF-β activity in the needful, can be performed.

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